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PREFACE

The literature in the field of microbiology is replete with examples of the ever increasing importance of viruses as causes of infectious diseases. Since the recent introduction of quantitative tissue culture methods for the detection of viruses, there has been a constant flow of papers describing new micro-organisms the listing and classification of which have not yet been completed. Improved methods are being developed to further our understanding of familiar viruses as well as of those recently discovered. In many instances investigators are undecided as to whether a new virus should be considered a harmless parasite or a pathogen, and, if the latter, whether it is the agent of a [illegible] or a new infectious disease. Medicine has [illegible] h agents are being [illegible].

To m[illegible] Progress in Medical Virology" is being [illegible] a new international series. It will include surveys that will cover etiologic, diagnostic, epidemiologic as well as basic laboratory aspects of virology. Virology no longer can be regarded only as adjunct of microbiology and clinical pathology, for it has developed into a science of its own. Those who learn the discipline of this science will find that through it they may probe into other areas, into studies of cells in the whole animal and in tissue culture, into studies of genetics, and into studies of cancer.

The Editors consider themselves fortunate to have secured the collaboration of outstanding investigators who have contributed much to recent developments in virology in different parts of the world. Thus each contribution provides a reliable survey of a current development. We sincerely hope that the "Progress in Medical Virology" will prove useful not only to our colleagues but also to others interested in this field.

We are indebted to our Publishers and especially to H. Karger, Ph. D., M. D. (hon.) for his help and support of this series.

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PRÉFACE

La littérature actuelle démontre l'importance toujours plus grande attribuée aux virus dans l'étiologie des maladies infectieuses. Depuis l'introduction des méthodes quantitatives d'explantation tissulaire des travaux toujours plus nombreux ont enrichi nos connaissances sur de nouveaux microorganismes, dont la description et la classification sont loin d'être terminées. Le perfectionnement constant des méthodes de travail permet en outre une différenciation plus poussée des virus connus et la découverte de nouvelles espèces. Dans ces conditions le chercheur est souvent indécis pour décider si un nouveau virus doit être considéré comme un parasite sans importance ou s'il représente l'agent pathogène d'une maladie infectieuse déjà connue ou celui d'une nouvelle maladie. La médecine paraît entrer dans une nouvelle phase, du moment que les agents pathogènes sont souvent reconnus avant les entités morbides qu'ils déterminent

C'est dans le but de mettre à la disposition des chercheurs des revues générales annuelles que les «Progrès en Virologie médicale» sont publiés. Cette nouvelle série groupera sur une base internationale des études d'ensemble sur les problèmes étiologiques, diagnostiques, épidémiologiques et sur les méthodes de travail en virologie. La virologie, en effet, ne peut plus être considéré comme un appendice de la microbiologie ou de la biologie médicale, elle est devenue une branche nouvelle des sciences médicales. Elle a ouvert de nouveaux chemins à l'étude des cellules dans l'organisme-même et en dehors de celui-ci, elle a permis également d'approfondir nos connaissances en matière de génétique et de carcinologie.

Les éditeurs sont heureux d'avoir obtenu la collaboration de chercheurs renommés dont les propres travaux ont contribué de façon décisive au développement de la virologie, c'est pourquoi le lecteur trouvera dans les volumes annuels des travaux correspondant aux plus hautes exigences Nous espérons que les «Progrès en Virologie médicale» seront utiles à nos collègues tout comme à ceux qui, de près ou de loin, s'intéressent au développement de l'étude des virus

Nous tenons à remercier les éditeurs, et plus spécialement Mr. H. Karger, Dr. ph , Dr med h. c., d'avoir compris nos idées et de nous permettre de les réaliser.

E. Berger
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VORWORT

Die einschlägige mikrobiologische Literatur ist voll von Beispielen über die zunehmende Bedeutung der Viren als Ursache von Infektionskrankheiten. Die Einführung der quantitativen Gewebekulturmethoden bei dem Nachweis der Viren löste eine Flut von Arbeiten mit Angaben über neue Mikroorganismen aus, deren Einteilung und Katalogisierung noch unvollendet ist. Infolge der ständigen Verbesserung der Nachweismethoden werden bekannte Viren schärfer differenziert und andere Viren neu entdeckt. Dabei stösst die Beantwortung der Frage, ob ein Virus als ein harmloser Parasit oder als ein Erreger einer Infektionskrankheit zu qualifizieren ist, häufig auf Schwierigkeiten. Als weitere Folge der Entdeckung von Viren ergab sich, dass neue klinische Einheiten zusammenzufassen sind.

Die letzten Ergebnisse der Virologie sollen in den jährlich erscheinenden Bänden «Fortschritte der medizinischen Virologie» gesammelt werden, welche als ein neues internationales Publikationsorgan erscheinen werden. Dieses dient der laufenden Übersicht, in welcher die Ätiologie, Diagnose und Epidemiologie, sowie die Laboratoriumsmethoden der Virologie zur Darstellung gelangen. Bei der Auswahl der Themen liessen sich die Herausgeber von dem Gedanken leiten, dass die Virologie heute nicht mehr ein blosses Teilgebiet der Mikrobiologie und klinischen Pathologie darstellt, sondern einen selbständig gewordenen Zweig der Wissenschaft. Er ermöglichte auf neuen Wegen das Studium der Zellen im Organismus und in der Gewebekultur, sowie ein tieferes Eindringen in die Genetik und die Wachstumsvorgänge des Krebses.

Die Herausgeber waren in der glücklichen Lage, führende Forscher als Mitarbeiter zu gewinnen, die durch eigene Studien zu der neuen Entwicklung der Virologie beigetragen haben. So besteht die Gewähr, dass die Beiträge dem letzten Stand der Wissenschaft entsprechen. Wir hoffen daher, dass die «Fortschritte der medizinischen Virologie» nicht nur den engeren Fachkollegen, sondern allen virologisch interessierten Kreisen von Nutzen sein werden.

Wir sind dem Verlag und insbesondere Herrn Dr. Dr. med. h. c. H. Karger für wertvolle Ratschläge und alle Hilfe zu Dank verpflichtet.

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Colter, J. S., Nucleic Acid as the Carrier of Viral Activity. Progr. med. Virol., vol. 1, pp. 1-35
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NUCLEIC ACID AS THE CARRIER OF VIRAL ACTIVITY

By JOHN S. COLTER

The first example of a direct biological activity of an isolated nucleic acid was the demonstration that bacterial deoxyribonucleic acid (DNA) could transmit a specific biological property possessed by the donor strain to a susceptible strain not possessing that property. This phenomenon of transformation has been the subject of recent reviews by HOTCHKISS (24, 25) while ZAMENHOF (46) has discussed the chemical and physical properties of the transforming principles. It is believed that the transforming deoxyribonucleates contain biologically specific entities equivalent to bacterial genes, and it has been shown by LERMAN (26) and by FOX (10), using P^{32}, that the number of cells transformed is directly proportional to the quantity of transforming DNA incorporated into cells of the receptor strain.

Ribonucleic acid (RNA) has long been implicated as the key substance in protein synthesis. The status of this question has been summarized by CHANTRENNE (4) and by BRACHET (2), and more recently has been the subject of a comprehensive review by SPIEGELMAN (41).

p-bromophenylisocyanate, and carbobenzoxy, chlorobenzoyl, and benzenesulfonyl chlorides. Both groups found that extensive substitution of the amino, phenol and indole groups could be effected with no resultant loss in infectivity. MILLER AND STANLEY (30) estimated that up to 70 per cent of the amino groups and from 10 to 20 per cent of the phenol and indole groups of the virus could be substituted without a decrease of activity. Of equal significance was the observation that the progeny produced by the infection of tobacco leaves with these derivatives were identical with normal TMV. These workers, had they possessed the terminology of today's geneticists, might reasonably have concluded that the protein moiety of TMV played little part in the "transmission of genetic information".

The next significant development in the continuing efforts to define the roles of TMV protein and RNA came with the observation by SCHRAMM and coworkers (32, 37, 38) that at a pH about 10, TMV disintegrates to yield protein particles with a molecular weight of about 90,000. They demonstrated further that these protein particles (A protein) could be made to reaggregate below pH 6.0 to give rise to rods of the same shape and size as TMV. All attempts to demonstrate infectivity of these protein rods were unsuccessful (32, 38). It might be noted here that this is the one area in which there is no disagreement between the numerous investigators of TMV. All agree that the A protein, by itself, is noninfectious.

More detailed studies of the alkaline degradation of TMV by SCHRAMM ET AL. (37) revealed that when the virus was exposed to a pH of 10.3 for a short period of time, a mixture of A protein, RNA and nucleoproteins was obtained. These nucleoproteins had molecular weights intermediate between that of TMV and of the A protein, and contained a higher percentage of RNA than did the intact virus particles. In electronmicrographs of such mixtures, SCHRAMM and his collaborators observed rods of TMV with gaps where only a thin filament of RNA could be seen (37). Protein fragments with a central hole were also found in the mixtures. These findings were confirmed by similar investigations of HART (20).

From these observations, TMV was pictured as a protein shell around a central core of RNA. That this picture is somewhat oversimplified is evident from the X-ray diffraction studies of FRANKLIN (15). These studies provided strong evidence that in the intact virus particle, the RNA does not be very close to the particle axis, but is deeply embedded in the virus protein. FRANKLIN concludes that the

The purpose of the present review is to summarize available information regarding still another biological property of nucleic acids, namely, the infectivity exhibited by viral nucleic acids. Activity in this rapidly developing area of research has been stimulated in large measure by the work of investigators at the Max-Planck-Institut für Virusforschung in Tübingen, Germany, and the Virus Laboratory, University of California, Berkeley, California, U.S.A. An attempt will be made here to summarize the extensive investigations which have been carried out on the tobacco mosaic virus (TMV). Studies of mammalian viruses, which received their impetus from the development by the Tubingen group of a new technique for the isolation of RNA, will be described and, finally, highly suggestive if not definitive studies with bacteriophage will be mentioned.

Experiments with Tobacco Mosaic Virus: Infectivity of TMV Ribonucleic Acid, and the Phenomenon of Reconstitution

It is proper that a review on the infectivity of viral nucleic acids should start with a discussion of the extensive investigations on TMV. It is proper not only because the work has provided the basis for studies on the infectivity of other viral nucleic acids, but because with this virus alone has it been possible to state unequivocally that the infectious nucleic acid demonstrated was obtained from intact virus particles. Since TMV was first obtained in a pure state by STANLEY in 1935, investigators have had at their disposal an elegant system for studies of the role of nucleic acids in the process of viral multiplication. Not only can the virus be obtained in a pure state, but it can be obtained in quantity, making it a system ideally suited to the correlation of biological function with molecular structure. These are advantages not generally available to workers in the field of mammalian viruses.

The first investigations on purified TMV were concerned with the function of the protein part of the virus. In the light of present knowledge it is easy to recognize that the earliest studies, performed in the laboratories of Schramm and of Stanley, indicated the central role played by ribonucleic acid in the replication of this agent. SCHRAMM AND MÜLLER (34, 35) and MILLER AND STANLEY (29, 30) prepared a number of derivatives of TMV, including those resulting from reactions between the virus and ketene, phenylisocyanate,

These preparations were shown to be inactive when tested separately at concentrations up to 1800 and 52 γ/ml of protein and RNA respectively. However, when 1 per cent solutions were mixed in the proportion of 10 parts protein to 1 part nucleic acid and incubated at 3° C. for 24 hours in the presence of acetate or phosphate buffer, pH 6.0–7.0, nucleoprotein particles carrying virus activity were formed. It was possible to dilute the mixture and assay directly. The more common practice was to separate the nucleoprotein by ultracentrifugation, to redissolve the pellet (which contained most of the protein and 40–60 per cent of the RNA) and remove traces of insoluble material by centrifugation before testing. Assays performed in *Nicotiana glutinosa* plants demonstrated that the nucleoprotein particles produced lesions at concentrations of 10–100 γ/ml which were indistinguishable from those produced by TMV at a concentration of 0.1 γ/ml.

The formation of active particles could not be shown when more dilute solutions of protein and nucleic acid were mixed, or when the complete reaction mixture was diluted 10-fold 1 minute after the addition of the buffer which triggered the aggregation. The capacity of the nucleic acid to give rise to infectious particles when mixed with the protein preparation was lost if it was first treated with ribonuclease (RNAse) or merely held for several weeks at 3° C. and pH 5.0.

Electron microscope studies of the protein and nucleic acid preparations and of the nucleoprotein particles formed from them proved illuminating. First, no TMV rods were found in the protein or the RNA when these preparations were examined at concentration levels 30–300-fold greater than those at which the reconstituted particles were assayed. In the nucleoprotein preparation on the other hand, rods were seen which appeared to be identical in shape and size with TMV except for a greater randomness in length. In their most active preparations, FRAENKEL-CONRAT AND WILLIAMS (14) found that about 1/10 of the particles, representing approximately ⅓ of the total mass, had lengths close to 300 mμ, the length of the monomer of TMV.

The application of HART's technique (20) of detergent treatment followed by electron microscope examination also provided useful information about these nucleoprotein rods. They proved somewhat more labile to sodium dodecyl sulfate than was TMV. However, after 10 seconds treatment, many rods were partly degraded and showed a central strand of material protruding from the ends – a picture remarkably similar to that obtained with TMV after a 60

nucleic acid seen as a core in the electronmicrographs of partially degraded TMV is in a collapsed form.

More pertinent to the present discussion than an elaboration of molecular configuration, however, was the observation made by SCHRAMM AND COWORKERS (36, 37) that these particles which had lost part of their protein were still infections. The infectivity of these nucleoprotein particles was at least 10 per cent that of the intact virus. Similar observations were made by HART (21) with TMV from which part of the protein had been removed by brief treatment with the detergent Duponol C. It was thus clear that at least a part of the virus protein is not necessary for infectivity, and the question of whether infectivity could be demonstrated after complete removal of protein became a very live issue.

Convincing evidence that this could indeed be shown was soon forthcoming from Schramm's laboratory. However, in the interests of a logical development of this story, the intriguing reconstitution experiments of FRAENKEL-CONRAT and of COMMONER should first be discussed.

FRAENKEL-CONRAT AND WILLIAMS (14) first described the formation of infectious particles from the inactive protein and nucleic acid components of TMV. The methods employed by these two workers for the preparation of the two components are described briefly below.

Protein component: TMV was dialyzed against pH 10–10.5 glycine buffer (0.01 M) or pH 10 5 carbonate-bicarbonate (0.1 M) at 3°C. for 48–72 hours. Undegraded virus was sedimented by ultracentrifugation, and the supernatant was brought to 0.4 saturation with ammonium sulfate. The protein alone precipitated, leaving the nucleic acid in solution. After two additional precipitations with 0.25–0 35 saturated ammonium sulfate, the protein solution was dialyzed, brought to pH 7.0–8.0 with NaOH and again subjected to high speed centrifugation.

Ribonucleic acid fraction: The virus solution (1 per cent) containing 1 per cent sodium dodecyl sulfate was adjusted to pH 6.5 and held at 40°C. for 16–20 hours. The solution was then made 0.35 saturated with respect to ammonium sulfate, and the protein which precipitated was removed by centrifugation. When the supernatant was refrigerated overnight, 60 to 90 per cent of the nucleic acid precipitated and was collected by centrifugation. It was further purified by repeated cycles of dissolution in ice-cold water and precipitation by the addition of two volumes of ethanol plus a few drops of 3 M pH 5 acetate. A final ultracentrifugation removed any trace of intact virus.

material was then removed by ultracentrifugation (104,500 g for 1 hour) and the resulting pellet was taken up in phosphate buffer for assay on leaves of *N. glutinosa*.

The assays performed by these workers were particularly well controlled. The separate components were tested for activity after treatment in a manner identical with the schedule employed with the protein-nucleic acid mixtures, including the polymerization step. In addition, protein-nucleic acid mixtures treated in an identical manner, except for the omission of the polymerization step, were examined for activity. Although the controls, unlike those reported by FRAENKEL-CONRAT AND WILLIAMS, contained some infectivity, a significant increase in infectivity was always found in the protein-nucleic acid mixtures polymerized by means of ammonium sulfate. The mixtures gave lesion counts 3 to 10 times greater than the combined infectivities of the control samples. As LIPPINCOTT AND COMMONER pointed out, it is difficult to imagine an increase in infectivity of this magnitude to be due to chance variation in the response of separate *N. glutinosa* leaves to infection with TMV.

COMMONER ET AL. (9) have reported on more extensive studies of the kinetics of this reconstitution, and have described a method which yielded particles of much higher specific activity than those obtained in their earlier studies. In brief, it consisted of adding the protein in small amounts, and over an extended period of time, to a relatively low concentration of virus RNA dissolved in a medium (28 per cent or more ammonium sulfate) in which the protein is relatively insoluble. They reported the formation, by this method, of reconstituted particles which approached 1/10 the infectivity of TMV and which produced about 1000 times as many lesions as did the uncombined starting materials.

Intriguing as the reconstitution experiments described in the foregoing paragraphs are, they do not go very far toward clarifying the specific role of the nucleic acid in the process of viral replication. Perhaps the only conclusion that can be drawn from them is that the infectivity of TMV is a joint property of RNA and protein, and that the infectivity regained in mixtures of the two components results from specific recombination of the two components into nucleoprotein identical with the virus. More recent reconstitution experiments described by FRAENKEL-CONRAT, however, are much more revealing, and bring us one step closer to the conclusion that the nucleic acid alone carries the virus' biological activity.

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The fact that a nucleoprotein is formed when TMV protein and RNA are mixed is not in itself surprising. RNA from any source shows a strong tendency to combine with basic proteins regardless of their origin. The pertinent question is whether the TMV components combine in some specific manner. Do they, as FRAENKEL-CONRAT AND WILLIAMS suggest, combine to yield particles identical or very similar to TMV? The answer seems to be that to some degree they do.

The nucleoproteins described by these workers contained 5–6 per cent RNA – as do TMV particles. The electron microscope studies, particularly those performed after detergent treatment of the reconstituted particles, are highly suggestive. It might be argued that the RNA *was itself infectious, and the protein, merely by combining* randomly with it, protected it from destruction by tissue enzymes or other factors, and made possible the demonstration of that activity. However, several workers in the field of mammalian viruses have reported that the presence of protein – as serum – inhibited the activity of infectious RNA preparations. The most reasonable explanation of this effect is that the active RNA was immobilized by a nonspecific combination with one or more of the serum proteins. In the present case, a reasonable premise is that the formation of infectious particles requires the formation of a higher polymer of RNA, possibly of molecular weight 2–3 $\times$ 10^6, and the fitting of this RNA with its associated protein in a highly specific manner. It might well be that all of the TMV protein and RNA in the preparations described by FRAENKEL-CONRAT AND WILLIAMS were potentially capable of forming infectious units, but that only a small per cent combined to give particles with a complete RNA "core" and with the required structural relationship between nucleic acid and protein.

Confirmation of the reconstitution experiments of FRAENKEL-CONRAT AND WILLIAMS came quickly from the independent investigations of LIPPINCOTT AND COMMONER (27). These workers employed the method of SCHRAMM ET AL. (36) to isolate TMV protein, and a modification of the method of COHEN AND STANLEY (5) to prepare the nucleic acid. The protein and nucleic acid preparations were mixed immediately and polymerization induced by the addition of solid ammonium sulfate to 0.4 saturation. After incubation for 1–6 hour periods at 2°C. insoluble material was removed by centrifugation and redissolved in pH 7.0 phosphate buffer. High molecular weight

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Intriguing as the reconstitution experiments described in the foregoing paragraphs are, they do not go very far toward clarifying the specific role of the nucleic acid in the process of viral replication. Perhaps the only conclusion that can be drawn from them is that the infectivity of TMV is a joint property of RNA and protein, and that *the infectivity regained in mixtures of the two components results* from specific recombination of the two components into nucleoprotein identical with the virus. More recent reconstitution experiments described by FRAENKEL-CONRAT, however, are much more revealing, and bring us one step closer to the conclusion that the nucleic acid alone carries the virus' biological activity.

In two separate communications, FRAENKEL-CONRAT (11) and FRAENKEL-CONRAT AND SINGER (12) have described mixed reconstitution experiments with protein and nucleic acid fractions isolated from different strains of TMV. Four strains of virus were employed – the common TMV, the masked (M), yellow aucuba (YA), and Holmes ribgrass (HR). The procedures for the isolation of the protein and RNA, and the conditions employed for reconstitution were essentially the same as those previously described (14). Active rods were successfully reconstituted from (1) TMV-nucleic acid and M-protein, (2) M-nucleic acid and TMV-protein, (3) YA-nucleic acid and TMV protein, (4) YA-nucleic acid and M-protein, (5) HR-nucleic acid and TMV-protein, (6) TMV-nucleic acid and HR-protein, and (7) HR-nucleic acid and M-protein. The infectivity of these mixed virus preparations was within the same range as that obtained from TMV-nucleic acid + TMV-protein.

Attention was drawn by FRAENKEL-CONRAT AND SINGER (12) to the fact that mixed virus preparations containing HR-RNA were found to be as active as those containing RNA from TMV. Since the original HR virus showed only 5 per cent of the activity of TMV on a weight basis, these workers interpreted their observation to indicate that the low infectivity of the HR strain was due to the protein component. If one accepts this explanation, however, it is very difficult to understand why the mixed virus composed of TMV-RNA and HR-protein was found to have comparable activity to that formed from TMV-RNA and TMV-protein

The serological characteristics of mixed virus preparations were shown to resemble those of the protein component. Anti-TMV serum was found to neutralize the mixed virus HR-RNA + TMV-protein to a similar extent as it did TMV, but to have little effect on the mixed virus TMV-RNA + HR-protein. Conversely, anti-HR serum neutralized the mixed virus containing HR-protein more effectively than it did the mixed virus containing TMV-protein. Though this observation can scarcely be called surprising, it does indicate that the immunological specificity of these virus strains is determined by their protein moieties alone. A direct method of appraising the contribution of nucleic acid to the antigenic properties of TMV might be to compare, on the basis of specificity and neutralizing capacity, antiserum prepared with SCHRAMM's A protein as antigen with antiserum prepared against an equivalent quantity of TMV.

From the point of view of this review, the most significant data coming out of these mixed reconstitution experiments concerned the nature of the disease caused by the synthetic viruses, and the nature of the progeny produced.

When each of the reaction products was tested in *Nicotiana tabacum* and *Nicotiana sylvestris*, it gave in every case the same symptoms as did the strain supplying the RNA. The mixed viruses TMV-RNA + M- or HR-protein gave a green mosaic disease, M-RNA + TMV protein produced virus without visible symptoms, mixed virus which contained YA-RNA gave a yellow mosaic disease, while those which contained HR-RNA produced typical ringspot lesions.

The mixed viruses formed from the protein and RNA components of TMV and HR were of particular interest in that the HR protein differs from TMV protein in amino acid composition and in antigenic specificity. Complete amino acid analyses were carried out on the progeny produced in the plant after infection with the two hybrid viruses. In each case the protein of the progeny closely resembled that of the virus from which the nucleic acid was obtained. The presence of about 0 7 per cent histidine and about 2 per cent methionine was demonstrated in the HR strain and in progeny resulting from infection with the mixed virus HR-RNA + TMV protein These amino acids were absent from TMV and from progeny of the mixed virus which contained TMV-RNA.

These mixed reconstitution experiments make the conclusion that nucleic acid is the sole genetic determinant of TMV almost obligatory. It would appear that the nucleic acid alone determines the nature of the disease produced as well as the biological and immunological properties of the progeny. However, a dissenting voice has been raised.

WANG AND COMMONER (44) have reported that under certain conditions they have been able to prepare an infectious nucleoprotein from TMV protein and DNA isolated from leaves of uninfected *N. tabacum* Although these workers reported that the initial infection produced in *N. glutinosa* by the DNA-containing nucleoprotein and by TMV could be differentiated on the basis of the number of lesions troduced on fresh leaves by the progeny of each, they showed that phe progeny of the infectious DNA-containing nucleoprotein were indistinguishable from ordinary TMV. The suggestion that a protein can exert structural effects upon a nucleic acid with which it is associated, thereby bestowing on said nucleic acid biological properties which it normally does not possess, is certainly an exciting one.

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Table I. Comparison of the Infectivity of Ribonucleic Acid and Tobacco Mosaic Virus in 0.1 M Phosphate Buffer.

pH	Ribonucleic Acid		Tobacco Mosaic Virus	
	μgm/ml	lesions	μgm/ml	lesions
6 1	10	153	0 09	95
			0 8	445
7 3	10	815	0 27	1048
7.3	1	524	0 05	795
7 5	10	998	0 27	685

From GIERER, A., and SCHRAMM, G. Nature *177*. 702 (1956)

GIERER AND SCHRAMM were unable to detect protein in their infectious RNA preparations by chemical methods, and showed by serological techniques that such preparations contained less than 0.02 per cent of TMV protein. Moreover, infectivity was not significantly reduced by repeating the extraction process with phenol. In addition to these observations, several other experiments excluded the possibility that the infectivity of the RNA preparations was due to contamination with intact TMV virus or residual parts of the protein shell.

Treatment of RNA and TMV with normal rabbit serum caused a reduction of the infectivity of both preparations. TMV antiserum, however, while causing no greater reduction in the infectivity of RNA than did the normal serum, almost completely abolished that of the virus. The infectivity of the RNA preparation, but not that of the virus, was abolished by brief treatment with ribonuclease (2 μgm/ml 4°C 10 mins), and by incubation at 20°C. *for 48 hours. After centrifugation* of the RNA solution for 30 mins. at 50,000 r.p.m. the infectivity of the supernatant was essentially unchanged. Identical treatment of the virus preparation resulted in the sedimentation of almost all the infectious particles. The results of these experiments, which seem to this reviewer to represent incontrovertible proof that TMV-RNA is itself infectious, are summarized in Table II.

RNA, isolated from TMV by sodium dodecyl sulfate, has also been reported to be infectious. FRAENKEL-CONRAT's first report (11) was

However, a final judgement on the validity of these observations must await further experimental evidence.

Although FRAENKEL-CONRAT (11) noted that when the TMV-RNA used in his reconstitution experiments was assayed at very high concentrations, lesions were occasionally produced which could not be attributed to the presence of intact virus particles, the first convincing evidence that TMV-RNA was itself infectious was provided by GIERER AND SCHRAMM (18, 19).

These investigators described a new method for the isolation of RNA from TMV, the importance of which cannot be overemphasized. Not only has this technique made possible such studies as have been done with infectious nucleic acids from mammalian viruses, but it will undoubtedly find wide application in investigations of the metabolism and biological properties of mammalian cell RNA. The method, elegant in its simplicity, the speed (approximately one hour) with which it can be accomplished, and in the fact that it gives essentially quantitative recovery of RNA, is described below.

Isolation of Tobacco Mosaic Virus RNA (Gierer and Schramm): A solution of 10 per cent TMV in 0.02 M phosphate buffer of pH 7.3 was shaken for 8 mins. at 5°C. with an equal volume of water-saturated phenol. The aqueous phase which contained the ribonucleic acid was separated by centrifugation and the process of extraction with phenol was repeated at least twice for 2 min. periods. The phenol was then extracted by ether from the aqueous phase.

When GIERER AND SCHRAMM inoculated leaves of *N. glutinosa* with RNA solutions prepared in this manner, and with standard TMV preparations, they found that 10 μgm of RNA produced about the same number of lesions as did 0.2 μgm of virus. Data obtained from these experiments are shown in Table I.

On a total weight basis, the infectivity of the RNA preparations described in this paper were of the order of 2 per cent that of the native virus. Expressing the infectivity of the RNA as a per cent of the infectivity of the same quantity of RNA as intact virus, values of 0.13 to 0.20 are obtained. More recently, SCHRAMM AND GIERER (33) have reported somewhat higher values. Data from 10 separate experiments have given average values for the infectivity of RNA of 4.5 per cent that of the same weight of virus, and 0.3 per cent that of the same quantity of RNA embedded in the virus particle.

in sufficient quantity to account for the observed infectivity, could not be found in their preparations, nor could the activity be attributed to the presence of intact virus rods. The activity of their RNA preparations was rapidly destroyed by concentrations of RNAse which were without effect on the intact virus, and an amount of anti-TMV γ-globulin which largely neutralized the infectivity of TMV did not appreciably affect that of RNA.

The data presented by GIERER AND SCHRAMM and by FRAENKEL-CONRAT AND HIS COLLABORATORS have raised a question which has remained unresolved – the question of the size of the infectious unit of RNA. On this point, the two groups are in sharp disagreement.

SCHRAMM AND GIERER (33) and GIERER (17) have presented evidence that the infectious component in their preparations has a sedimentation velocity of the order of $S_{20} = 20S$, and have calculated from sedimentation and intrinsic viscosity measurements that the active unit has a molecular weight of approximately 2×10^6. Since the whole RNA content of a single TMV particle corresponds to a molecular weight of 2–3×10^6, these workers have suggested that biological activity depends upon the integrity of the RNA core. The relationship between infectivity, intrinsic viscosity and mean molecular weight is shown in Table III, in which are summarized the

Table III Decrease of intrinsic viscosity (η), mean molecular weight (weight average) m, and infectivity, L, with time. The decrease was induced by $1.5 \times 10^{-3} \mu$ gm/ml ribonuclease at 22°C. in a solution of 0.16 per cent ribonucleic acid in 0.02 M phosphate buffer, pH 7. The infectivity, L, is given by the number of lesions per 25 leaves of *Nicotiana glutinosa* inoculated with 20 μgm/ml ribonucleic acid in 0.1 M phosphate.

t (min.)	[η] (cc/gm)	m	L (lesions)
0	300	1.20×10^6	980
5	274	1.10×10^6	786
11	254	1.02×10^6	383
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22	214	0.86×10^6	272
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37	168	0.67×10^6	62
65	115	0.46×10^6	1

From GIERER, A.: Nature *179*: 1297 (1957)

Table II. Comparison of Ribonucleic Acid (10 μgm/ml) and Tobacco Mosaic Virus (0.27 μgm/ml) in 0.1 M Phosphate Buffer of pH 7.3 (Infectivity expressed as lesions per 30 leaves).

	Ribonucleic Acid	Tobacco Mosaic Virus
Normal	488	629
With normal serum	180	117
With antiserum	145	0
With ribonuclease	0	473
After ultracentrifugation	367	31
After 48 hrs. at 20°C	2	130

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not accompanied by substantiating experimental evidence, and although he did comment on the failure to find virus rods in the nucleic acid preparation, on its marked instability and its lability to RNAse, it was not possible to compare the relative activities of his RNA and TMV preparations.

More recently, FRAENKEL-CONRAT, SINGER AND WILLIAMS (13) have published the results of a more detailed study on infectious RNA prepared by the detergent method. From these data, and from data included in a report by SIEGEL ET AL (40) on the ultra-violet inactivation of infectious RNA, it has been possible to get some idea of the activity of TMV-RNA isolated by this method. The RNA described by the latter workers contained 0 006 per cent the infectivity of a quantity of TMV containing the same weight of the nucleic acid. Applying the same calculations to the data supplied by FRAENKEL-CONRAT AND CO-WORKERS, values are obtained which range from 0.007 for the least active to 0.05 per cent for the most active preparation, with most values falling in the lower portion of the range. When it is recalled that similar values for GIERER AND SCHRAMM'S preparations were 0.1 to 0.5 per cent (with a mean of 0.3 per cent), the conclusion is inescapable that the phenol method yields much more active RNA than does the detergent method.

Nevertheless, FRAENKEL-CONRAT'S group has provided convincing evidence that their RNA preparations are, per se, infectious. Protein,

in sufficient quantity to account for the observed infectivity, could not be found in their preparations, nor could the activity be attributed to the presence of intact virus rods. The activity of their RNA preparations was rapidly destroyed by concentrations of RNAse which were without effect on the intact virus, and an amount of anti-TMV γ-globulin which largely neutralized the infectivity of TMV did not appreciably affect that of RNA.

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The nucleic acid preparations of FRAENKEL-CONRAT'S group, on the other hand, had a sedimentation velocity of $S_{20} = 6S$–$10S$, which could represent a polynucleotide chain of molecular weight 200–300,000. These investigators rejected, on the basis of centrifugation experiments, the suggestion that the activity of their preparations resided in a minor component of high molecular weight. They concluded that the activity was associated with the bulk of the material, and shared with it a molecular weight of about 250,000.

These differing opinions are difficult to reconcile. A possible explanation, albeit a rather tenuous one, is that the degradation of TMV-RNA by means of sodium dodecyl sulfate produces fragments on some of which are located all the sites necessary for biological activity, while spontaneous or RNAse mediated depolymerization produces quite different fragments, none of which contain all the centers required for activity. Also difficult to reconcile with the concept of an active RNA fragment of molecular weight about 250,000 is the conclusion, reached by both FRAENKEL-CONRAT and COMMONER, that in reconstitution experiments, activity was associated only with those particles which closely resembled TMV particles in shape and size, and which presumably contained a complete nucleic acid core.

Another puzzling aspect of the TMV story is the failure of SCHRAMM AND CO-WORKERS to demonstrate the phenomenon of reconstitution with their phenol-prepared RNA, particularly since this material possesses significantly greater biological activity than do nucleic acid solutions prepared by the detergent method. However, the information provided by FRANKLIN (15) regarding the intimate structural relationship between protein and nucleic acid in TMV prompts certain speculations. If, as seems probable, active nucleoprotein particles are formed from TMV protein and RNA only when certain strict structural requirements are fulfilled, it is not difficult to imagine that this can happen only when protein and nucleic acid helices are built up simultaneously from relatively small building blocks. Attempts to form active virus particles from protein and phenol-prepared RNA could be likened to attempts to insert the structural steel into a building after it, in all other respects, was completed.

Infectious Ribonucleic Acid from Mammalian Viruses

In the short interval which has elapsed since GIERER AND SCHRAMM described their technique (the phenol method) for the isolation of infectious RNA from TMV, a total of five RNA preparations capable of initiating mammalian virus diseases have been described. The viruses involved have been those of Mengo, West Nile and Eastern equine encephalitides, and Types I and II poliomyelitis.* Except in the case of Type I poliomyelitis, no steps were taken by the investigators concerned to purify the viruses prior to the isolation of RNA. In each case the method employed for the preparation of the infectious nucleic acid was that of GIERER AND SCHRAMM.

The isolation of infectious RNA from cells infected with Mengo, West Nile, and Type II poliomyelitis (MEF1 strain) viruses has been described by COLTER and co-workers (6, 7). The Mengo and West Nile viruses were grown in cells of the Ehrlich ascites carcinoma, while the poliomyelitis virus was propagated in the central nervous system tissue of suckling hamsters. Details of these investigations are described in the following paragraphs.

The Ehrlich ascites carcinoma was grown in 18–20 gram Swiss albino mice, and was infected in the seventh day of tumor growth by the intraperitoneal injection of a saline suspension of virus-infected mouse brain. For the West Nile and Mengo viruses, 0.5 ml of 1:20 and 10^{-4} dilutions were employed respectively. Infected cells were collected 120 hours following the administration of the West Nile virus and 60–68 hours after infection with the virus of Mengo encephalitis The cells were washed several times in saline before being rapidly frozen in a mortar set in an alcohol-dry ice bath. Hamster brains and spinal cords were harvested 18–24 hours after the intracerebral injection of 0.03 ml of a 10 per cent suspension of polio-infected hamster brain, and were immediately and rapidly frozen.

The frozen tissues were ground by hand to fine, homogeneous powders, which were then mixed intimately (Waring blendor) with 0.14 M saline – 0.01 M Na citrate, pH 7 0, to give 10 per cent (w/v) suspensions. The suspensions were centrifuged at 3000 r.p.m. for

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* HAYS, SIMMONS and BECK (Nature *180* 1419, 1957) suggest that a mixture of DNA and RNA isolated from leukemic and nonleukemic spleens and lymph nodes of AKR mice may, when injected into newborn $C3H_f$ × AKR F1 hybrid mice, cause an increased incidence of leukemia in the adult animals

20 mins. and the supernatants carefully removed. Extracts so prepared were considered to be 10^{-1} dilutions of the viruses.

Ribonucleic acid was separated from such virus preparations by the phenol method. The virus preparations were extracted three times by vigorous shaking with an equal volume of water-saturated phenol. After each extraction the aqueous phase was separated by a brief centrifugation. Traces of phenol were removed from the aqueous phase by several ether extractions and the ether was evaporated by bubbling nitrogen gas through the solution. All manipulations were carried out at 0–4° C. Ribonucleic acid solutions prepared in this way were designated as 10^{-1} dilutions for convenience in comparing the relative infectivities of virus and ribonucleic acid preparations. It should be pointed out that this method of comparison is valid only if RNA is extracted quantitatively from the virus particles present in the crude virus preparations. *Infectivity titers*, expressed as LD_{50}, were determined in 10–12 gram Swiss mice by the intracerebral injection of 0.03 ml of serial 10-fold dilutions.

In Tables IV and V are listed the infectivities of a number of Mengo, West Nile and poliomyelitis virus preparations, and of ribonucleic acid solutions prepared from them. Infectious RNA could

Table IV Infectivity of Mengo Encephalitis Virus Preparations and of Ribonucleic Acid Isolated from Them.

Hours post Mengo Infection	LD_{50}*	
	Mengo Virus Preparation	Ribonucleic Acid
48	6.6	2.5
48	5 5	2.5
48	4 5	1.6
52	4 5	1.4
56	5.4	2.4
64	5.8	2.5
60	5 5	2.5
64	5.8	2.5
68	6.2	2.5
72	6.2	2.8

* Expressed as logarithms to the base 10.

From COLTER, J. S., BIRD, H H., and BROWN, R. A.: Nature *179*: 859 (1957)

Table V. Infectivity of West Nile, Poliomyelitis and Bunyamwera Virus Preparations, and of Ribonucleic Acid Isolated Therefrom.

Virus	LD_{50}*	
	Virus Preparation	Ribonucleic Acid
Poliomyelitis	5 5	2 0
	6 4	2 3
	6 4	2 4
	5.8	2 2
West Nile	5 4	2.5
	5 4	2 6
	5 5	2 4
	5 0	2 5
Bunyamwera	4 8	—**
	5 4	—**

* Expressed as logarithms to the base 10

** No mice died at 10^{1} dilution.

From Colter, J S, Bird, H H, Moyer, A. W., and Brown, R. A.: Virology 4 522 (1957)

be prepared routinely from Ehrlich ascites tumor cells infected with Mengo or West Nile viruses and from MEF1 poliomyelitis virus-infected hamster brain and spinal cord. The infectivity of the ribonucleic acid preparations from all three virus-infected tissues was of the order of 0.1 per cent that of the virus suspensions from which they were prepared.

The *ribonucleic acid preparations contained* no protein which could be detected by the biuret reaction. Furthermore, paper chromatography following acid hydrolysis failed to demonstrate amino acids. The nitrogen-phosphorus ratios were 1.6–1.7, and all the phosphorus could be accounted for as ribonucleotide phosphorus. However, in view of the low degree of infectivity of the RNA preparations compared to that of the corresponding virus suspensions, and the relative insensitivity of the techniques for detecting protein as compared with those for estimating infectivity, failure to detect

Table VI. Comparison of Mengo Encephalitis Virus and Ribonucleic Acid Preparations

	LD_{50}*	
	Mengo Virus Preparation	Ribonucleic Acid
Control	6.5	2.6
After ribonuclease	6 6	—**
After 6 hrs at 37° C	6 4	—**
Control	5 8	2.8
After ribonuclease	5 8	—**
After 6 hrs at 37° C.	5 8	—**
Control	6 6	2 6
1 After centrifugation (top 9 ml)	3 5	2 2
Control	7 2	2 5
1 After centrifugation (top 4 ml)	4.5	2.0
After centrifugation (middle 4 ml)	4 5	2 5
Control	7.0	3 3
2 After 1 M sodium chloride	4.5	3.1
3 After 1 M sodium chloride	3.5	3.5

* Expressed as logarithms to the base 10.
** No mice died at 10^{-1} dilution

1. 10 ml samples, 30,000 r.p.m , 60 mins.
2. Pellet washed once with ice-cold 1 M NaCl
3. Pellet washed twice with ice-cold 1 M NaCl,

From COLTER, J S., BIRD, H H., and BROWN, R. A Nature *179* 859 (1957)

protein was not considered sufficient grounds for ruling out the possibility *that the measured infectivity was due to contamination* with intact virus particles. The conclusion that, in each case, the infectivity was due to a ribonucleic acid component, was based on evidence obtained from four different types of experiments. They are described briefly below. Data obtained therefrom are illustrated in Tables VI, VII and VIII.

The infectivities of the RNA preparations were abolished by brief treatment with *ribonuclease* (10 γ/ml, 15 mins. 20° C) and by incuba-

Table VII Comparison of West Nile Encephalitis Virus and Ribonucleic Acid Preparations

	LD_{50}*	
	Virus Preparation	Ribonucleic Acid
Control	5 0	2.4
After ribonuclease	4 5	—**
After 6 hrs at 37° C.	4 5	—**
Control	5 5	2.5
After ribonuclease	5 5	—**
After 6 hrs at 37° C.	5 0	—**
Control	5 5	2.4
After 1 M NaCl	2 3	2 4
Control	5 4	2.5
After 1 M NaCl	3 0	2.8
Control	5.5	2 4
1 After centrifugation (top 4 ml)	0 8	0 8
After centrifugation (middle 4 ml)	2 3	1 5
Control	5 0	2 5
2. After centrifugation (top 4 ml)	2.4	1.6
After centrifugation (middle 4 ml)	2.7	2 0

* Expressed as logarithms to the base 10
** No mice died at 10^{-1} dilution

1 30,000 r p m , 60 mins
2 25,000 r p m , 60 mins.
From COLTER, J S., BIRD, H H , MOYER, A W , and BROWN, R. A.. Virology 4 522 (1957).

tion at 37° C. for 6 hours. Neither treatment had any significant effect on the infectivities of the corresponding virus suspensions.

The intact virus particles and the infectious components in the corresponding RNA preparations behaved quite differently in 1 M sodium chloride solution. Aliquots of each were made 1 M with respect to sodium chloride and were incubated for 12–16 hours at 0° C. before being centrifuged at 3000 r.p.m. for 20 minutes. The pellets were

Table VI. Comparison of Mengo Encephalitis Virus and Ribonucleic Acid Preparations.

	LD_{50}*	
	Mengo Virus Preparation	Ribonucleic Acid
Control	6.5	2.6
After ribonuclease	6.6	—**
After 6 hrs at 37° C.	6.4	—**
Control	5.8	2.8
After ribonuclease	5.8	—**
After 6 hrs at 37° C.	5.8	—**
Control	6.6	2.6
1. After centrifugation (top 9 ml)	3.5	2.2
Control	7.2	2.5
1. After centrifugation (top 4 ml)	4.5	2.0
After centrifugation (middle 4 ml)	4.5	2.5
Control	7.0	3.3
2. After 1 M sodium chloride	4.5	3.1
3. After 1 M sodium chloride	3.5	3.5

* Expressed as logarithms to the base 10.
** No mice died at 10^{-1} dilution.

1. 10 ml samples; 30,000 r.p.m., 60 mins.
2. Pellet washed once with ice-cold 1 M NaCl.
3. Pellet washed twice with ice-cold 1 M NaCl.

From COLTER, J. S., BIRD, H. H., and BROWN, R. A.: Nature *179*: 859 (1957).

protein was not considered *sufficient* grounds *for ruling* out the possibility that the measured infectivity was due to contamination with intact virus particles. The conclusion that, in each case, the infectivity was due to a ribonucleic acid component, was based on evidence obtained from four different types of experiments. They are described briefly below. Data obtained therefrom are illustrated in Tables VI, VII and VIII.

The infectivities of the RNA preparations were abolished by brief treatment with ribonuclease (10 γ/ml, 15 mins. 20° C) and by incuba-

Finally, the infectious components of the virus and ribonucleic acid preparations could be differentiated on the basis of their sedimentation velocities. Centrifugations were carried out in a Spinco ultracentrifuge, employing a no. 40 rotor. The usual procedure was to centrifuge 10 ml aliquots of each preparation, and to determine separately the infectivities of the top and middle 4 ml of each. The virus particles were shown to sediment more rapidly than did the infectious components in the corresponding RNA preparations.

Mengo, West Nile and Type II poliomyelitis viruses were recovered from the brains of mice which died following the intracerebral injection of the corresponding ribonucleic acid preparation. In each case, identification was accomplished by means of specific immune serum.

ALEXANDER ET AL. (1) have reported the isolation of infectious RNA from concentrated, partially purified Type I poliomyelitis virus. Their *preparations reproducibly produced* plaque *formation* and confluent cytopathogenic areas on HeLa cell monolayers. Type I poliomyelitis virus was identified as the end product of the infection initiated by RNA. Typical data are shown in Table IX.

These investigators reported that the cytopathogenic action of their RNA preparations was completely inhibited by RNAse at a final concentration of 100 γ/ml, but not by the same concentration of DNAse, papain or chymotrypsin. The infectivity of the RNA was also

Table IX. Infectivity of Type I Poliovirus RNA in HeLa Cell Monolayers.

Exp.	No Exposure to RNAse Before Seeding			Exposure to RNAse 1 min. before seeding		
	No of Plates	Dilution After pH Adjustment	Average No of Plaques/Plate	No of Plates	Dilution	Number of Plaques/Plate
1	10	none	40 + confluent area	8	½	0
	5	¼	1 2 (1–2)*			
2	14	none	confluent area	16	none	0
	12	1/10	4 (0–14)*			
3	14	none	confluent area	12	none	0
4	10	none	confluent area	10	none	0
5	8	none	40 + confluent area	15	none	0

* Numbers in parentheses show variation among plates.

From ALEXANDER, H. E., KOCH, G., MOUNTAIN, I. M., SPRUNT, K., and VAN DAMME, O.: Virology (in press)

Table VIII. Comparison of Poliomyelitis Virus and Ribonucleic Acid Preparations.

	LD_{50}*	
	Virus Preparation	Ribonucleic Acid
Control	5.5	1.5
After ribonuclease	5.7	—**
After 6 hrs at 37° C.	5.4	—**
Control	5.5	2.4
After ribonuclease	5.5	—**
After 6 hrs at 37° C.	5.0	—**
Control	6.4	2.4
After 1 M NaCl.	2.5	2.3
Control	6.4	2.3
After 1 M NaCl	3.5	3.0
Control	6.4	2.4
1. After centrifugation (top 4 ml)	4.0	1.5
After centrifugation (middle 4 ml)	4.0	2.4
Control	6.0	2.2
1. After centrifugation (top 4 ml)	4.3	2.2
After centrifugation (middle 4 ml)	5.0	2.5

* Expressed as logarithms to the base 10

** No mice died at 10^{-1} dilution

1. 30,000 r.p.m., 60 mins.

From COLTER, J. S., BIRD, H. H., MOYER, A. W., and BROWN, R. A.: Virology *4*: 522 (1957).

washed in ice-cold 1 M sodium chloride and then resuspended in saline to their original volumes and their infectivities determined. The pellet obtained from each virus suspension contained but a small fraction of the infectivity of the original extract. In general, recovery – in terms of infectivity – was of the order of 0.1 per cent. With each of the RNA preparations, however, the infectivity was recovered quantitatively in the 1 M sodium chloride pellet.

Table X. Infectivity of Virus and RNA after Various Methods of Treatment.

Material	VIRUS			RNA		
	LD_{50} Mouse	LD_{50} Egg	*PFU*	LD_{50} Mouse	LD_{50} Egg	*PFU**
Original	>9.5	—	—	3.77	—	—
With RNAse	>9.5	—	—	Ø	—	—
Centrifuged	6.77	—	—	2.02	—	—
Original	>9.5	6.69	9.30	4.52	1.80	Ø
With RNAse	>9.5	—	—	Ø	—	—
Centrifuged	>6.5	—	—	2.26	—	—
Original	—	6.21	9.00	—	2.15	+
With RNAse	—	6.04	8.30	—	Ø	Ø
Centrifuged	—	4.69	6.34	—	0.89	—
Alcohol precipitated	—	<3.00	<3.00	—	2.23	+
4.5 hrs. at 37°C.	—	5.49	8.65	—	Ø	—

LD_{50} Calculated by the method of REED AND MUENCH Am. J. Hyg. *27* 493 (1938)
— Indicates sample not tested
Ø No demonstrable infectivity.
* Plaque counts are not given because of the described irregularities

From WECKER, E., and SCHÄFER, W. Z. Naturforsch. *12b* 415 (1957)

washings in a buffer-ethanol (1:2) mixture, was resuspended in buffered saline to the original volume. The virus lost all demonstrable activity while the infectivity of the RNA was recovered quantitatively. Table X summarizes the results of these studies.

More recently, WECKER AND SCHÄFER* have successfully isolated infectious RNA from EEE-infected chick embryos. The method employed for the isolation was identical with that used in the studies of RNA from EEE-infected mouse brains. Embryos killed by EEE infection were quickly frozen. Four frozen embryos were homogenized with 12 ml of 0.02 M Na phosphate, pH 7.0 and 20 ml of water-saturated phenol. The rest of the procedure was the same as that previously described.

* The author wishes to thank Dr. EBERHARD WECKER for making available the data discussed here prior to its publication in Z. Naturforschung.

completely inhibited by monkey serum at 1/10 dilution whether it was pooled normal or Type I antiserum. A similar observation has been made by COLTER ET AL (6). Plaque formation by RNA was not prevented by rabbit normal gamma-globulin or anti-Type I gamma-globulin. The latter did, however, at a concentration shown to neutralize whole active poliovirus, cause a reduction in the size of the plaques produced by RNA, presumably by limiting the spread of intact virus particles from the initial center of infection. Neither normal monkey serum nor RNAse reduced the number of plaques formed by whole poliomyelitis virus.

Infectious RNA has been isolated by WECKER AND SCHÄFER (45) from mouse brains infected with the virus of Eastern equine encephalitis (EEE). These workers found that it was necessary to modify slightly the method of GIERER AND SCHRAMM in order to obtain active nucleic acid. They removed the brains from mice which had died of EEE and froze them rapidly at —40°C. The frozen brains were homogenized with a 1:1 mixture of 0.14 M NaCl–0.01 M Na phosphate, pH 7.0 and water-saturated phenol in the proportion of 2 ml of mixture per brain. The aqueous phase obtained after centrifugation for 10 minutes at 1350 r.p.m. was extracted three additional times with phenol. Traces of phenol were removed with ether, and the ether evaporated with N_2 in the usual manner.

An infectious principle could be demonstrated regularly in these RNA preparations employing either the mouse or the embryonated egg as the test system. Attempts to demonstrate infectivity by the formation of plaques in monolayers of chick embryo fibroblasts gave erratic results. RNA, isolated from EEE-infected mouse brains which had been homogenized in buffer prior to the addition of phenol, was found to be inactive.

Evidence showing that the infectious agent in the RNA preparations was a species of RNA and not contaminating EEE virus was obtained from experiments similar to those reported by GIERER AND SCHRAMM (18, 19) and by COLTER ET AL. (6, 7). Thus, the infectivity of the RNA was destroyed by RNAse and by incubation at 37°C. Neither treatment had a marked effect on the infectivity of the virus control. The sedimentation velocities of the infectious components in the RNA and virus preparations were shown to differ. In addition, a marked difference in the behavior of the two agents in ethanol was demonstrated. Aliquots of virus and RNA preparations were added to two volumes of ethanol, and the precipitate in each, after two

Table XII. The Effect of RNAse and DNAse on the Infectivity of a Nucleic Acid Fraction (Incubation for 15 mins at 10° C.)

Treatment	LD_{50}/ml
Control	2 86
RNAse 0 01 γ/ml	0
DNAse 1 0 γ/ml	3 45
DNAse 0 1 γ/ml	3 13
DNAse 0 01 γ/ml	3 39

From WECKER, E., and SCHÄFER, W.: Z. Naturforsch. (in press)

tion of the material with DNAse destroyed the DNA peak seen in the ultracentrifuge pattern and increased its activity somewhat (Table XII). Precipitation at 66 per cent ethanol, which destroyed the virus completely, also caused a slight increase in the activity of the nucleic acid preparations.

WECKER AND SCHÄFER have suggested, on the basis of these observations, that high molecular weight DNA and amino acids and/or peptides are capable of inhibiting the biological activity of the RNA. However, these workers showed that approximately 40 per cent of the RNA in their preparations was not precipitated in 66 per cent ethanol. COLTER (unpublished data) found that low molecular weight RNA, when added to solutions of RNA isolated from Mengo-infected Ehrlich ascites tumor cells, significantly reduced the infectivity of the latter. Therefore, it seems reasonable to suggest that the inhibitory substances removed by ethanol precipitation from WECKER AND SCHÄFER's preparations were low molecular weight ribonucleic acids.

The infectious material which multiplied in embryonated eggs following the injection of active nucleic acid preparations was shown by WECKER AND SCHÄFER to be intact EEE-virus. It was neutralized by EEE-antiserum, was resistant to the action of RNAse, and was *destroyed by precipitation with ethanol.*

The three groups, whose work is described in the foregoing pages, appear to have no major differences of opinion. In each case, the conclusion that an infectious RNA had been isolated was based on very similar – if not identical – experimental evidence. There seems

The nucleic acid fractions obtained from chick embryos in this manner contained about 60 per cent RNA and 40 per cent DNA plus some unidentified sugars in a highly polymerized state and some nitrogen-containing substances which gave a positive biuret test. WECKER AND SCHÄFER concluded that the latter were amino acids or small peptides, since they remained in solution when the infectious RNA was precipitated in 66 per cent ethanol. Ultracentrifugal analyses of the preparations revealed two RNA peaks (S_{20} = 24 S and 16 S at a total nucleic acid concentration of 0.1 per cent) plus a DNA peak, the identity of which were established by treatment of the nucleic acid preparations with RNAse and DNAse. The preparations were infectious to 10–11 day old embryonated eggs (Table XI). They also produced plaques in monolayers of chick embryo fibroblasts. However, as was the case with RNA from EEE-infected mouse brains, the number of plaques produced was not proportional to the quantity of RNA placed in contact with the cell monolayers. ALEXANDER ET AL. (1) have made similar observations with infectious RNA isolated from Type I poliovirus.

The characteristics of this infectious RNA were identical to those of the RNA from EEE-infected mouse brain. Its biological activity was destroyed by RNAse and incubation at 37°C., and was inhibited by both normal horse serum and by horse anti-EEE serum. Incuba-

Table XI. The Infectivity of Virus Preparations and Nucleic Acid Fractions for 10 to 11 Day Old Embryonated Eggs.

No.	LD_{50}/ml	
	Virus Preparation	*Nucleic Acid Fraction*
1	8.1	2 9
2	7.3	3 3
3*	6 5	3 1
4*	7.7	3.7
5*	6 3	3.2
Average	7.2	3.3

* The nucleic acid fractions were purified by alcohol precipitation

From WECKER, E., and SCHÄFER, W.: Z. Naturforsch. (in press).

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Treatment	LD_{50}/ ml
Control	2 86
RNAse 0 01 γ/ml	0
DNAse 1 0 γ/ml	3.45
DNAse 0.1 γ/ml	3 13
DNAse 0 01 γ/ml	3 39

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no reason to doubt that in each case the RNA initiated an infection which led to the formation of intact virus particles identical to thoes from which the infectious nucleic acid was isolated. Nevertheless, there are minor points on which there is not complete unanimity. In addition there are observations, made by all three groups, which are puzzling and which need clarification.

WECKER AND SCHÄFER reported that when mouse brains or chick embryos were homogenized in buffer prior to extraction with phenol, the RNA obtained was biologically inactive. COLTER ET AL. (6, 7), on the other hand, experienced no difficulty in isolating active RNA from poliomyelitis virus-infected hamster brain and spinal cord or from Mengo or West Nile-infected Ehrlich cells when these tissues were homogenized and centrifuged prior to the addition of phenol. In fact, infectious RNA was obtained on several occasions from saline homogenates of polio-infected hamster brain and cord which had been stored at −20°C for several months. The suggestion that the release of cellular RNAse by homogenization is responsible for the failure to obtain active RNA is scarcely tenable. The resistance of the intact viruses to the action of this enzyme – as compared with the extreme lability of the corresponding RNA preparations – has been the pivotal evidence provided regarding the identity of the infectious component in the RNA preparations. Moreover, it may be reasonably assumed that any RNAse present is rapidly inactivated by phenol.

The presence of DNA in the nucleic acid preparations isolated by *WECKER AND SCHÄFER from EEE-infected chick embryos is puzzling.* Though ALEXANDER ET AL. did not report on the chemistry of their active preparations, COLTER and co-workers showed that in their three infectious preparations, all the phosphorus could be accounted for as ribonucleotide phosphorus, and that the diphenylamine reaction for DNA was consistently negative. Furthermore, COLTER (unpublished data) has employed the phenol extraction procedure to isolate *RNA from Ehrlich ascites tumor cell nuclei suspended in* buffered physiological saline, and found that no DNA was left in the aqueous phase. It is possible that the failure of phenol to sediment the DNA of chick embryos quantitatively is a reflection of some property unique to chick embryo DNA, or perhaps indicates the presence in chick embryos of considerable quantities of low molecular *weight DNA unassociated with protein. WECKER AND SCHÄFER found* that the DNA in their preparations exhibited a sedimentation velocity

(at zero concentration) of 10S, a value which seems very low for this nucleic acid. The tonicity of the aqueous phase may be important. In the case of the EEE-infected chick embryos, the aqueous phase was 0.02 M phosphate buffer, whereas the EEE-infected mouse brains were homogenized with 0.14 M NaCl – 0.01 M phosphate buffer and phenol. It may be significant that WECKER AND SCHÄFER made no reference to the presence of DNA in the latter preparations. It is to be hoped that future investigations will explain this difference in the findings of these two groups.

If the premise that the immunological specificity of a virus is the property of its protein moeity is accepted, a reasonable assumption would be that antiserum prepared against an intact virus would not inhibit the activity of the protein-free RNA isolated from that virus. The fact that immune serum was found by all three groups to inhibit the infectivity of active RNA would have been very damaging but for the fact that normal serum was found to inhibit the activity with equal efficiency. In an unexpected way these observations showed that the infectious components in the viral and RNA preparations were different, since the infectivity of the virus was, in all cases, inhibited only by the specific immune serum However, the mechanism by which serum – immune or normal – inhibits the activity of RNA remains obscure ALEXANDER ET AL. (1) suggested that the inactivating effect of serum may be due to the presence of trace amounts of RNAse, and their observation that neither rabbit normal gamma-globulin nor anti-Type I poliomyelitis gamma-globulin inhibited plaque formation by their RNA preparations appears to strengthen this contention However, WECKER AND SCHÄFER found that gamma-globulins from normal horse and horse anti-EEE sera, as well as the whole sera, inhibited the activity of RNA from EEE-infected chick embryos. Perhaps an answer to this question will be provided by the use of high potency antiserum. It might then be possible to demonstrate significant neutralization of the intact virus at serum dilutions at which the nonspecific inhibitory effect on RNA would be diluted out.

ALEXANDER and co-workers, and WECKER AND SCHÄFER have commented on the erratic behavior of their infectious RNA preparations in tissue culture With intact virus preparations the number of plaques produced on monolayers of susceptible cells is proportional to the dilution of the virus. This was not the case with RNA isolated from Type I poliomyelitis virus and tested on HeLa cell monolayers,

no reason to doubt that in each case the RNA initiated an infection which led to the formation of intact virus particles identical to thoes from which the infectious nucleic acid was isolated. Nevertheless, there are minor points on which there is not complete unanimity. In addition there are observations, made by all three groups, which are puzzling and which need clarification.

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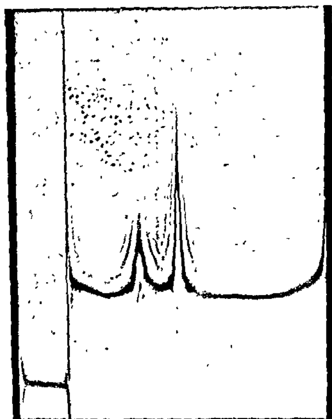


Fig 1. Analytical ultracentrifuge pattern of ribonucleic acid isolated from Ehrlich ascites tumor cells 16 minutes after rotor attained speed of 50,740 r p m.

virus was found to sediment more rapidly than did the intact Mengo and poliomyelitis viruses.

TIMASHEFF (43) has studied the 1 M NaCl-insoluble fraction of Ehrlich ascites cell RNA by means of light scattering, and has estimated the mean molecular weight of this fraction to be 1.2×10^6. These data, and those cited in the above paragraph, suggest that the molecular weight of infectious RNA from Mengo, poliomyelitis and West Nile viruses is of the order of $1.5\text{–}2.0 \times 10^6$. SCHWERDT (39) has estimated the molecular weight of the MEF1 poliomyelitis

or with RNA isolated from EEE-infected mouse brains or chick embryos and tested on monolayers of chick embryo fibroblasts. COLTER AND CO-WORKER have also reported on the irregularity with which infectious RNA from poliomyelitis-infected tissues produced lesions in monkey kidney tubes. There is no obvious explanation for this finding. Perhaps all that can be said at present is that whereas tissue culture systems are in general at least as sensitive as the intact animal for the detection and titration of virus agents, they appear to be less sensitive systems for the demonstration of the biological activity (infectivity) of RNA.

Although Type II (MEF1) poliomyelitis and Coxsackie viruses have been crystallized by SCHAFFER AND SCHWERDT (31) and by MATTERN AND DUBUY (28) respectively, quantities of crystalline mammalian viruses, adequate to permit the complete physical characterization of RNA derived therefrom, are not as yet available to investigators.

The infectious preparations described in this section consisted mainly of cellular ribonucleic acids with the infectious (viral) RNA species present in extremely small quantities. Nevertheless, from the data available certain tentative conclusions may be drawn regarding some of the physical properties of these infectious ribonucleic acids.

COLTER AND BROWN (8) have described the isolation of RNA from Ehrlich ascites carcinoma cells, and BROWN ET AL (3) have described a number of the physical properties of such preparations. Ultracentrifuge studies showed that at a concentration of 0.003 per cent, one third of this material exhibited a sedimentation constant of less than 3S, while the remainder, though there was some spreading of the boundaries, sedimented as two components, 60 per cent with S=32 and 40 per cent with S=15 (figure 1). Essentially identical ultracentrifuge patterns were obtained with RNA preparations from Ehrlich cells infected with Mengo and West Nile viruses and with RNA from polio-infected hamster brain The S=15 and S=32 components sedimented quantitatively at 1 M NaCl concentration, and with infectious preparations the infectivity was always recovered quantitatively in the 1 M NaCl pellet. Centrifugation experiments on these infectious RNA preparations suggested that the infectious species had sedimentation constants of the order of S=32. The data suggested further that the infectious RNA from West Nile virus-infected cells was larger than that from either the Mengo or polio-virus infected tissues. It is interesting to note that the West Nile

infectivity. Heated (71°C. 15 mins.), as well as unheated shockates, were employed in these studies.

Heated shockates, which contained less than 10^2 infectious particles/ml as determined by plaque counting with intact cells, produced infectious T2 virus when incubated with protoplasts of *E. coli* B. Increases as high as 10^9 plaque-forming units/ml in 60 mins. at 37°C. were observed. Phage increases were also demonstrated when unheated shockates were incubated with *E. coli* B protoplasts. Neither incubation of shockates with intact *E. coli* cells nor of intact T2 virus with *E. coli* protoplasts resulted in the formation of new virus particles.

The production of virus in protoplast-shockate mixtures required the presence of sucrose and amino acids, and was inhibited by versene (0.01 M) and by potassium cyanide, potassium arsenite and potassium arsenate at 0.001 M concentration or higher. When heated shockates were incubated with trypsin (50 γ/ml, 30 mins.) and the action of trypsin inhibited by the addition of soybean trypsin inhibitor before mixing the shockate and protoplast preparation, 0.1 per cent as much phage was produced as in the absence of the trypsin treatment. DNAse had little if any effect on the ability of the shockates to produce phage. Unheated shockates were resistant to both trypsin and DNAse. Activity of heated shockates was destroyed by homogenization in a chilled Waring blendor, by agitation in a Mickle disintegrator, and by exposure to ultraviolet light under conditions which caused little inactivation of intact T2 phage.

Of particular interest was SPIZIZEN's observation that T2 shockates were capable of producing phage when incubated with protoplasts derived from certain bacterial species – *E. coli* B/2, anaerogenic *E. coli* (WR-2), atypical *Klebsiella* (WR-3), Providence group paracolon (WR-6) and *Aerobacter aerogenes* 417 – which are resistant to infection with the intact virus. The resistance of these species may be attributed to the lack of proper receptor sites. Protoplasts from other resistant strains, however, failed to support virus reproduction when incubated with T2 shockates. The nature of the resistance of these organisms is not known. However, a reasonable suggestion might be that they are deficient in some part of the metabolic machinery required for the synthesis of the viral components.

SPIZIZEN has suggested that the infectious particles in his shockates were DNA units protected by a protein shell. This picture is certainly compatible with the experimental evidence. Whether this protein

virus to be 6.8×10^6 and has found by chemical analysis that it contains 25–30 per cent RNA. From this data, the molecular weight of RNA from a single poliomyelitis virus may be calculated to be $1.7–2.04 \times 10^6$. That these values are in rather good agreement with those arrived at indirectly is quite evident, and leads this reviewer to suggest that the infectious ribonucleic acids isolated from Mengo, West Nile and poliomyelitis virus-infected tissues represented the complete, undegraded nucleic acid complements of these viruses. GIERER AND SCHRAMM, it should be recalled, reached the same conclusion in the case of TMV-RNA.

Studies with T2 Bacteriophage

The central role played by T2 virus deoxyribonucleic acid (DNA) in the reproduction of the virus has been evident for a number of years. HERRIOTT (22) showed that the protein coat of T2, isolated after osmotic rupture, adsorbed to the receptor site on the cell wall of the host (*Escherichia coli*) and stopped multiplication of the host cell without giving rise to new phage particles. The experiments of HERSHEY AND CHASE (23) demonstrated clearly that T2 DNA was associated with the replicating activity of the virus, and indeed suggested that it carried all the genetic information necessary for virus reproduction. These observations have raised the question as to whether the protein moiety of T2 virus is concerned only with the 'injection' of DNA or whether it plays some role in viral replication subsequent to the entrance of DNA into the host cell. The investigations of SPIZIZEN (42) and of FRASER and co-workers (16), to be discussed here, have provided a partial answer to this question. They have not, however, provided unequivocal evidence that protein-free T2 phage DNA can, by itself, be infectious.

SPIZIZEN (42) has shown that protoplasts, derived from *E. coli* B by the action of lysozyme, will support the production of new phage particles when incubated with T2 virus preparations disrupted by osmotic shock. Phage strain T2r + was propagated on *E. coli* B in a glucose-ammonium salts medium and concentrated by differential centrifugation. Phage concentrates, containing 2×10^{11} plaque-forming particles/ml in 3 M NaCl, were rapidly diluted 40-fold in 1 per cent gelatin solution resulting in 99 per cent or more reduction in

addition to protoplasts, they produced approximately 3 per cent as many initial plaques as did corresponding untreated samples.

In all experiments performed by FRASER and co-workers with T2 DNA preparations, controls were run with non-protoplasted cells, handled identically except for the lysozyme treatment. They found a small but persistent infection in this control. This infection was markedly reduced by DNAse, and it occurred with *E. coli* B/2 as well as with *E. coli* B, thereby differentiating it sharply from T2 infection. The authors concluded that this represented infection of *E. Coli* with their crude DNA and suggested that it was due to enhanced cellular permeability of *E. coli* caused by washing.

FRASER ET AL were conservative in their conclusions. They remarked on the remote possibility that the infecting material was T2 whose tail specificity had been altered by urea and whose DNA had been made susceptible to DNAse action, and pointed out correctly that the results did not exclude the possibility that protein was bound to the DNA. However, it is also possible that the infectious component in their preparations was protein-free phage DNA, and it is probably not overoptimistic to anticipate that unequivocal evidence in support of this premise will soon be forthcoming.

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may participate in the synthesis of virus particles, or be involved in attachment to the protoplast membrane, or merely prevent the formation of DNA aggregates too large to penetrate the protoplasts, is not yet clear.

The experiments of FRASER and collaborators (16) are even more suggestive than those of SPIZIZEN (42), and bring us closer to the conclusion that, in the case of T2 virus at least, DNA is the carrier of phage infectivity. Like SPIZIZEN, these investigators employed protoplasts derived from *E. coli* by means of lysozyme, but instead of osmotically shocked T2, they used DNA solutions prepared from concentrated virus by means of urea. Concentrated, purified T2 virus was diluted into concentrated (8 M in 0.1 M saline, pH 8.2) urea solution. After incubation for one hour at 37°C. the preparations were dialyzed against 0.1 M saline at 2°C. until free of urea. These preparations were slightly opalescent and contained no viable phage when plated directly on intact *E. coli*. Attempts to infect *E. coli* protoplasts with intact T2 virus also gave consistently negative results.

When DNA solutions, prepared as described above, were mixed with protoplasted *E. coli*, production of intact virus could be demonstrated routinely. Phage production was also demonstrated when these DNA preparations were incubated with protoplasts from the T2 resistant *E. coli* B/2, thus verifying the observations of SPIZIZEN. Blank platings of DNA, of experimental bacteria and protoplasts failed to produce plaques in *E. coli*. Evidence was presented to show that (a) the DNA preparations did not contain T2 masked by DNA, other degradation products or urea, (b) the yield of phage did not represent initially inactive but still intact plaque particles, reactivated in some way, and (c) the yield did not represent phage particles dissociated by urea into protein and DNA, and reconstituted by some action of the protoplasts.

When DNA-protoplast mixtures were shocked into water after adsorption, all infective centers were eliminated. Identical treatment of intact T2 virus did not reduce its infectivity. The effects of DNAse on the activity of the DNA preparations clearly differentiated them from T2 virus preparations, and from the T2 shockates studied by SPIZIZEN. Whereas the latter were little affected by DNAse, the activity of the DNA preparations of FRASER ET AL. (16) was largely eliminated by exposure to this enzyme. When DNA preparations were incubated with DNAse (10 γ/ml, 80 mins. 37°C.) before their

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lympthocytic choriomeningitis virus (TRAUB, 1938). Perhaps a middle portion of this spectrum is occupied by the virus of herpes simplex which causes a prolonged infection of man and is not detectable during periods when it is quiescent and not responsible for overt disease, but is readily isolated when it is provoked to induce localized cell damage (ANDERSON AND HAMILTON, 1949). As expressed in another way, virus may be infectious and persist, as may bacteria in the carrier state, or may exist in a true masked or noninfectious state.

The development of tissue culture techniques permitted the commencement of cultures of tissue fragments or individual cells from a wide variety of organs obtained from many different animals. In a number of instances, although the tissues were secured from apparently normal organs or from organs not obviously infected by virus, prolonged culture *in vitro* uncovered the presence of viruses which were cytopathic for the cultured cells. The evidence appears clear that the agents were present in the explanted tissues and were not introduced accidentally into the cultures. The experiments described presented additional evidence that viruses may persist and propagate in animals without induction of demonstrable disease. In each of the studies which have illustrated this point, queries have been made concerning the state of virus in the original host cells and the mechanism by which it persisted without detection.

Tissue culture appears to afford opportunities to investigate by qualitative and quantitative techniques various postulates to explain the mechanism by which virus may infect cells without cell injury and thus persist in the host for an indefinite period. It is the object of this review to describe examples whereby virus has been "unmasked" by *in vitro* cultures of tissues, to summarize and discuss experimental models of persistent viral infections or the carrier state in tissue culture systems, and to consider the implications of these findings.

Viral Carrier State in vivo

Prior to a consideration of the investigations which describe persistent viral infections of various cell lines in tissue culture, those studies will be reviewed which imply that a viral carrier state may exist in an intact host. In each instance, to detect virus it was necessary to culture the infected tissues by artificial means.

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THE SIGNIFICANCE OF THE VIRAL CARRIER STATE IN TISSUE CULTURE SYSTEMS

By HAROLD S. GINSBERG

Introduction

Nature furnishes a number of instances which exemplify viral infections of animals, plants, bacteria and insects without obvious manifestations of injury. These illustrate prolonged parasitism of cells by viruses without demonstrable harm to the host. Details of the persistent viral infections vary considerably according to the virus, the host, and the conditions. In some examples, cell injury is not usually observed in the infected host (TRAUB, 1936; THEILER, 1937), while in contrast viral infections have been described in which the agent is provoked to increased multiplication and production of disease (HORSFALL AND HAHN, 1940; ANDERSON AND HAMILTON, 1949). As the course of the infection varies with the examples described, so too the apparent state of the virus may differ. The best documented instances range from a true provirus state in lysogenic bacteria (LWOFF, 1953), to readily demonstrable virus in mice infected with

and were shown to be agents which produced eosinophilic intranuclear inclusion bodies in fibroblasts. These agents were subsequently identified to be strains of human salivary gland virus, and to be closely related to or identical with strains of this agent isolated by SMITH (1956) and WELLER ET AL. (1957).

Viruses from monkey kidneys. The widespread use of tissues from animals has further revealed the intriguing fact that certain apparently normal organs carry undetected viruses. The most notable of these, possibly because of its widespread usage, is the monkey kidney. RUSTIGIAN ET AL. (1955) observed that vacuolated syncytial structures containing groups of many cell nuclei appeared in some uninoculated cultures of cells from trypsinized monkey kidneys after incubation for 12 days or longer. All cells of the cultures were gradually altered in this manner, and these subsequently underwent complete degeneration. The cytopathic changes were demonstrated to be due to a transmissible agent, probably a virus. The agents described have subsequently been detected in uninoculated monkey kidney cultures by numerous investigators, but no specific disease of monkeys or other animals has been associated with them. Monkeys from whose kidneys the so-called "foamy agents" have emerged during *in vitro* culture have circulating neutralizing antibodies directed against the homologous virus.

Monkey kidneys appear to be particularly susceptible organs for viral carrier infections. In addition to "foamy agents" and adenoviruses previously discussed, at least 18 other viruses have been isolated from uninoculated cultures of seemingly normal monkey kidneys (HULL ET AL., 1956; HULL and MINNER, 1957; ROWE ET AL., 1958) These latter agents have been termed SV or simian viruses, and are distinquished by the viruses' immunologic or cytopathic characteristics.

To explain the mechanism by which these examples of persistent viral infections in animals were unmasked, the interpretation employed to explain adenovirus infections of tonsils and adenoids may be invoked: namely, that the virus is suppressed by the conditions existing within the host. When the environment is altered by transfer of the infected cells to *in vitro* culture conditions and a medium with-

and identified.

Adenovirus. The initial study to indicate clearly that host tissues may be infected, but evidence no clinical manifestations of viral infection and yield no detectable virus, was that of ROWE ET AL. (1953). These investigators noted spontaneous degeneration of epithelial-like cells growing from explants of adenoids and tonsils in long-term cultures. From these cultures, a group of related cytopathogenic agents, subsequently termed adenoviruses (ENDERS ET AL. 1957), was isolated and characterized. It is noteworthy that virus could not be isolated from homogenates of these tissues, and that patients from whom the tonsils and adenoids had been extirpated possessed circulating type-specific antibodies which could neutralize the virus isolated (HUEBNER, ET AL. 1954). These studies have subsequently been confirmed in a number of laboratories. From the data presented it is not possible to know the period adenoviruses had persisted in tonsil or adenoid nor the state of the virus, whether mature or incomplete, during the period in which it was carried. Analysis of the evidence available, however, suggests that adenoviruses infect more than 50 per cent of tonsils and adenoids removed surgically (HUEBNER ET AL., 1954; WINTER AND SCHLESINGER, 1956); that virus is masked or suppressed by type-specific antibody, and possibly other host factors, and that upon prolonged culture of the tissues in artificial media conditions for cell metabolism and growth are altered, antibodies are gradually removed and virus is permitted to emerge (HUEBNER ET AL., 1954; ROWE ET AL., 1955).

In addition to tonsils and adenoids, it would appear possible that cells of other lymphoid tissues may serve as natural hosts for carrier infections with adenoviruses. In at least two instances it has been reported that type 3 adenovirus was obtained in tissue culture from mesenteric lymph nodes (HUEBNER ET AL., 1954; KJELLEN, 1955).

It has been reported more recently that at least three distinct adenoviruses of higher types have been obtained from monkey kidney epithelial cells cultured in a monolayer for several weeks (HULL AND MINNER, 1957; ROWE ET AL., 1958). The mechanisms and implications appear similar to those described above.

Human salivary gland virus. Adenoids, perhaps as part of their natural functions, may serve as host for a number of inapparent viral infections. ROWE and coworkers (1956) during the course of studies on the infection of adenoids by adenoviruses in some explants, observed spontaneous degeneration of fibroblasts but not epithelial cells. Three similar strains of viruses were isolated by this technique,

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Viral Carrier State in Tissue Culture

The availability of numerous types of tissues and the increasing number of stable cell lines which can be propagated in continuous culture have afforded the opportunity to investigate the quantitative as well as qualitative details of viral multiplication and the cytopathology which may result. From these studies data have evolved to indicate that under certain circumstances viruses may persist in tissues for long periods, and, indeed, even in cells propagated in serial cultures. There are many examples of the multiplication of viral particles in cells of tissues without extensive cellular destruction. Many of these experiments, however, were not designed to investigate the means by which viruses may persist and multiply without extensive cellular injury, and consequently neither permit analysis nor allow speculation of the mechanism involved. Such studies, therefore, will not be considered although many have stimulated further work on the problem with which this discussion concerns itself. Emphasis will be placed upon investigations that offer experimental results which may lead to notions of the mechanisms by which the viral carrier state is established and maintained. The experimental models to be discussed are of several types. For the purpose of presentation they may be conveniently divided into three main groups consisting of viral carrier cultures which have been initiated in:
1) "Resistant" cells which comprised the majority of cells in the culture;
2) "Resistant" cells selected from a predominantly susceptible cell population by pressure of viral action; and 3) Susceptible cells.

1. Carrier Cultures in "Resistant" Cells

There are many examples to demonstrate that viruses may multiply in animals without causation of disease either because the viruses are avirulent or unadapted to the host, or because the animal is resistant by natural or acquired means. From these numerous examples it might be anticipated that viruses may multiply in cells *in vitro* without obvious pathological effect. BEARD AND ROUS (1929) with vaccinia virus demonstrated this possibility to be a fact. Subsequent observation of this phenomenon in prolonged infection in a single culture and in serial cultures has suggested the simplest form of a

viral carrier state. Although the mechanism by which viruses damage cells or, conversely, the means by which cells resist injury while supporting viral synthesis, is not understood, the concept of persistence of virus in tissues not damaged by its presence is now not a difficult one to accept.

The earliest studies to describe the persistence of virus in cultured tissues were with explants. One of the best documented was that by FELLER, ENDERS AND WELLER who described infection with and continued propagation of vaccinia virus in mixed embryonic chick tissues or fragments of chick heart for as long as nine weeks (FELLER ET AL., 1940). That viral infection of cells occurred was proved by demonstration of the development of typical cytoplasmic inclusion bodies in scattered cells. Despite the continued viral multiplication, infected tissue fragments grew to the same extent as those in control cultures, and could be readily explanted to fresh cultures The data presented implied that many of the cells infected resisted extensive injury and survived. Thus, vaccinia could persist and continue to infect new cells and multiply therein for the duration of the experimental period

The balance between viral action and tissue growth was accomplished with vaccinia virus and mixed chick tissues, although cellular changes could be detected and despite the fact that greater amounts of virus potentially could produce extensive cytonecrosis. BANG, LEVY AND GEY similarly noted propagation of fowl pox virus in chick fibroblasts without any detectable cell damage for as long as 120 days (1951). It has also been observed in a number of instances that poliovirus could multiply to a relatively high titer in certain cell lines in tissue culture with minimal or no cytopathic changes. These experimental results were dependent upon either a careful selection of viral variants (SABIN, 1954), or upon propitious choice of cell lines (LEDINKO ET AL., 1951; KAPLAN AND MELNICK, 1955; BARSKI, 1957). In one of these studies, KAPLAN AND MELNICK (1955) presented evidence that the continued presence of type 1 poliovirus in high titer was dependent upon infection of a relatively few susceptible cells (0 015 to 0.21 per cent) in a population of epithelial cells which were relatively resistant to the agent. That similar circumstances exist in many of the other described examples of viral multiplication without evident cytopathology seems possible.

These studies illustrate clearly the capacity of some cells in a culture to support viral propagation without the entire culture submitting to generalized disintegration by the parasite. This situation,

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1. Carrier Cultures in "Resistant" Cells

There are many examples to demonstrate that viruses may multiply in animals without causation of disease either because the viruses are avirulent or unadapted to the host, or because the animal is resistant by natural or acquired means. From these numerous examples it might be anticipated that viruses may multiply in cells *in vitro* without obvious pathological effect. BEARD AND ROUS (1929) with vaccinia virus demonstrated this possibility to be a fact. Subsequent observation of this phenomenon in prolonged infection in a single culture and in serial cultures has suggested the simplest form of a

influenza A viruses. The resistance to heterologous virus was transitory and disappeared in the rare instances in which cultures were "cured" of virus by passage in the presence of immune serum for as long as 4 months. Ordinarily when immune serum was added to culture fluid of the infected cultures virus could not be detected in either cells or fluid, but when serum was removed, virus was soon measurable again. The resistance to unrelated virus was not the result of an inability of the resistant cells to adsorb virus, and was considered to be an example of the interference phenomenon resulting from noninfectious virus in these refractory cells. The altered properties of the cells were not a genetic characteristic, for cloned lines derived from carrier cultures not only were free of virus but also were fully susceptible to vesicular stomatitis virus and could be made into carrier cultures again with NDV.

The data presented were interpreted on the assumption that only about 1 in 50 to 100 cells was receptive and able to support viral multiplication, perhaps due to the physiological or nutritional state of the cells. The other cells could adsorb virus, but did not permit synthesis of new viral particles. These latter refractory cells combined with virus induced a state of interference with a subsequent viral infection. This series of events continued until all cells either produced infectious virus or contained noninfectious virus and thus were resistant. The latter cells could divide, but the progeny were not resistant. The cycle was then repeated, as a small percentage of the progeny were receptive and the majority were refractive. This concept in certain aspects is in agreement with that suggested by Chambers (1957) to explain the phenomenon of persistent infection of L cells with western equine encephalomyelitis virus, as discussed elsewhere in this paper.

Another example of a viral carrier culture initiated in relatively resistant cells was established with Coxsackie A-9 virus in HeLa cells which support multiplication of this agent but are relatively insusceptible as compared to monkey kidney cultured cells (Takemoto and Habel, 1958). This model is basically similar to the NDV persistent infection of MCN cells discussed above. There are differences of sufficient importance, however, to warrant description of the carrier culture involved. Although HeLa cells are relatively resistant to A-9 Coxsackie virus, when a sufficiently large amount of virus was employed, cytopathic effects could be observed. Upon addition of fresh growth medium containing human, horse or calf serum, the cultures recovered so that no evidence of viral infection was detectable

which may be of considerable significance in the intact animal, has been confirmed by many other similar investigations.

The evidence that it is possible to establish a prolonged viral infection *in vitro in a culture in which the majority of cells resist the* lethal effects of virus suggested that stable cell lines with similar properties should offer the opportunity to effect an equilibrium between virus and cell which could be maintained indefinitely by continuous subculture of infected cells. This has now been accomplished with several viruses and cell lines.

Using two epithelial-like cell lines, originally obtained from the bone marrow of a leukemic patient and from a human embryonic lung, MCN and Lung-To respectively, (HENLE AND DEINHARDT, 1957), DEINHARDT, HENLE, BERGS AND HENLE (1957, 1958) *established* prolonged and persistent infections through numerous subcultures with three myxoviruses: Newcastle disease (NDV), mumps, and 6–6 an agent related to but significantly different from classical mumps virus (HENLE AND DEINHARDT, 1957). These agents can multiply in the cells employed, but cause no significant injury to the host cultures. The most extensive experiments were done with NDV in MCN cultures, and the essentials of these will be discussed. The results obtained with the other agents in MCN or Lung-To cells were similar whenever comparable experiments were performed.

The cell lines employed could be readily infected with any of the myxoviruses, and unless greater than 10 infectious doses of virus per cell was inoculated, no evidence of cell damage was discernible. Even when an excessive quantity of virus was used, the cytopathic effects were minimal and did not hinder subculture of the unaffected cells. The evidence indicates that subcultures could be made at 2 to 3 week intervals, apparently indefinitely, with continued propagation of virus as well as cells. The infectious virus in the cultures ranged from about $10^{2.0}$ ID_{50} to $10^{5.0}$ ID_{50}, and most was associated with the cells *rather than the fluid. Several lines of evidence indicated clearly that* relatively few of the cells in the persistently infected cultures actually were producing infectious virus (approximately 1 in 50 to 1 in 120). In spite of the fact that such a small proportion of cells actually yielded infectious virus, the cell cultures in general showed characteristics to indicate that the cells were different from MCN cells of uninfected cultures. The cells of the carrier cultures exhibited: 1) a decreased growth rate; 2) an increased aerobic glycolysis; and 3) a resistance to the unrelated vesicular stomatitis, herpes simplex and

BANG AND GEY (1952), who noted that cultures of certain strains of rat cells, both malignant and nonmalignant, survived despite the early destruction of a large number of cells by eastern equine encephalomyelitis virus The surviving cells appeared uninjured, and soon comprised the entire culture as far as could be determined visually. Virus persisted in reduced amounts in the apparently healthy cultures, but in some instances an increased propagation of virus occurred and extensive cell damage again ensued Such cycles were observed as many as 4 times in some experiments. These studies have been extended to demonstrate that factors which are favorable to vigorous cell multiplication, i.e. chick embryo extract and incubation at 37°C, foster the "recovery" of partially destroyed cultures and the development of a chronic viral infection (BANG ET AL, 1957). These experiments do not present evidence to indicate the proportion of cells infected or the state of susceptibility to viral infection of the majority of cells, although the authors suggest the hypothesis that the cells are not uniformly susceptible to viral infection and only a small proportion of the cells produce virus. In addition, the data presented in the latter study imply that cultural conditions which are conducive to rapid cell propagation and maintenance of cells in a state of vigor render partially resistant cells even less susceptible to the cytopathic effect of the virus of eastern equine encephalomyelitis.

The elegant experiments carried out by PUCK, CIECIURA and coworkers offer an intriguing example of a persistent viral infection in selected variant cells (CIECIURA, MARCUS AND PUCK, 1957; PUCK AND CIECIURA, 1957). An extensive infection of HeLa cells by Newcastle disease virus (NDV) resulted in survival of only an occasional cell. Those cells which resisted the destructive action of NDV were grown into colonies and the virus challenge repeated two or three times at weekly intervals. From these repeated destructive viral insults populations of cells were obtained which resisted NDV cytopathic effects. Clonal strains were derived from these NDV resistant cultures and passed in series. The cells thus obtained showed no evidence of viral infection and not only resisted viral challenge but also could propagate into colonies of cells with a plating efficiency which approached 100 per cent in the presence of as much as 12 viral particles per cell. This marked resistance of cells to NDV may be explained by the fact that these cells could no longer adsorb virus That the majority of cells in the resistant cultures are actually infected is suggested by the fact that from 64 to 100 per cent of the cells produce

despite the fact that virus was readily measured in the culture fluid. These cultures could be subcultured for many cell generations with consistent recovery of virus but no evidence of cell injury. When type-specific immune serum was included in the medium, virus was readily eliminated from the cultures. As further evidence that this carrier culture was not comparable to the lysogenic state in bacteria, clone isolates of cells (PUCK ET AL., 1956) from carrier cultures were free of virus. The data further suggested that not only was a small proportion of cells in carrier cultures infected, but also that infected cells did not divide and could not establish clones.

The data reviewed imply the ease whereby persistent viral infections can become established in cells which are neither uniformly nor completely susceptible to the virus employed. Indeed, the experiments of TAKEMOTO AND HABEL indicate that prolonged cultivation of carrier cultures selects cells with an increased resistance to Coxsackie A-9 infection as well as virus with altered properties. The evidence presented by these investigators indicates that one of the principal factors responsible for continued persistent infection of the carrier cultures is the relative insusceptibility of HeLa cells to Coxsackie A-9 virus, especially when cells are in a state of high metabolic activity promoted by growth medium. A second important factor is the failure to accumulate large quantities of virus in the cultures due to the rapid thermal inactivation of virus under conditions where virus propagates at a relatively slow rate.

2. *Carrier Cultures in Selected "Resistant" Cells*

That persistent viral infections or viral carrier cultures can be promoted in cultures of resistant cells has been amply confirmed. In the examples described it was noted that often cells in a given culture are not homogeneous in their response to viral infection. These illustrations were based upon viral carrier cultures established in cell lines in which a preponderance of cells were relatively insusceptible to infection. In addition, there are several noteworthy instances of viral carrier cultures initiated in cells selected from a population in which the majority of cells not only supported viral multiplication but also reacted to infection by severe injury. The cells which survived the viral cytopathogenic effect were then relatively resistant to injury by the agent. An early example of this phenomenon was suggested by

poliovirus antibody was essential for continued propagation of the altered cells in serial subculture. Upon degeneration of the cells in the absence of specific antibody, poliovirus could be detected in most cultures. The identical series of events could be initiated by exposing HeLa cells to a large amount of virus for 1 hour, after which virus was removed by washing the cells, and a culture fluid containing type-specific immune serum was added. Most of the cells underwent necrotic changes, but a small percentage of the original population survived and could then be cultured in series if poliovirus antibody was present in the culture fluid. The data indicated that not only did HeLa cells multiply in the presence of virus, but also that virus multiplied as well, albeit at a low level. After antibody was removed, viral multiplication commenced at a more rapid rate following a long delay period (ACKERMANN, 1957).

It must be emphasized that in the poliovirus carrier cultures, although cells multiplied to an extent that serial subcultures apparently could be made *ad infinitum*, the cells obtained were decidedly changed from the wild type HeLa cell from which these cultures were derived. They were altered not only with respect to morphology, but also in their oxygen consumption and cytopathic response to a second viral infection. Thus the cells of such carrier cultures appeared spindly and elongated and had a reduced quantity of cytoplasm relative to nuclear size; their oxygen consumption was decreased; and although they could be superinfected with another type of poliovirus or with Coxsackie virus, the resultant cell degeneration developed at a slower rate and viral multiplication had a longer lag phase than in the parent cell line (ACKERMANN, 1957a). In the most recent communication, ACKERMANN (1957b) states that the altered cell line retains resistance to viruses, Coxsackie as well as poliomyelitis virus, even after being freed from the carrier agent with the use of trypsin in addition to antibody. Cells which are completely resistant to poliovirus have not been obtained, however. Thus, although it has not been determined whether cells which are infected can propagate, nor what is the precise role played by virus during the carrier state, it does appear that the cells involved are genetically altered and that specific antibody is required to restrain viral multiplication without complete disintegration of all cells in the cultures. The exact nature of the interactions among cell, virus and antibody, and the mechanisms involved in the phenomenon described have been only partially elucidated. Whatever the mechanisms involved are, however, the model described may have

plaques when plated upon sensitive "giant cell" cultures derived from x-ray-irradiated HeLa cells (PUCK AND MARCUS, 1955). The infectious state of NDV within the cells, however, is not clear and data have not been presented to indicate whether virus which will infect the parent HeLa cells or chick embryos can be obtained directly from the resistant cells. When resistant cells were cultured in the presence of NDV immune serum for about 3 weeks the cells lost the capacity to induce plaques when plated onto irradiated cells, but did not lose their resistance to infection by NDV. Subsequent culture in antiserum-free medium permitted the gradual return of infected cells. Thus, although the viral carrier state is not strictly comparable to lysogenic bacteria, these studies do imply the development of an intriguing relationship between virus and a genotypic-variant HeLa cell. Subsequent experiments will undoubtedly clarify certain puzzling aspects of this model as well as elucidate further its mechanism.

3. *Carrier Cultures Established in Susceptible Cells*

The examples discussed above described the development of viral carrier cultures in either naturally resistant or selected resistant cells. In nature, it is apparent that recurrent viral infections as a result of a latent infection may be established in cells fully susceptible to the injurious effects of the agent. Thus, under certain circumstances, virus persists and probably multiplies, but cells are not damaged; an alteration of the conditions may make the cells fully susceptible to cytonecrosis by virus and disease is manifest. It might therefore be postulated that viral carrier cultures can be established in cells fully susceptible to infection when conditions are properly arranged and controlled. Such has proved to be the case under varying circumstances: 1) presence of specific antibody; 2) presence of non-specific serum inhibitors of virus; 3) serum factors which maintain cells under conditions of optimum metabolism; 4) nutritional deficiency of cells; and 5) viral interference by noninfectious virus. An example of each of these will be discussed.

ACKERMANN AND KURTZ (1955) observed that one of several lines of HeLa cells in their laboratory had an unusual morphology and underwent spontaneous degeneration when maintained in the absence of certain pools of human serum. It became clear that poliomyelitis virus was responsible for the cytonecrosis, and that type-specific

showed a significant alteration in susceptibility to subsequent infection with homologous or heterologous types of adenoviruses.

Because neutralizing antibody was present in most human sera, the role of antibodies in this phenomenon was investigated. Extensive experiments indicated that a large quantity of neutralizing antibody in rabbit serum, in the absence of human serum, was not adequate to maintain the infected cultures without progressive cytopathic changes. On the other hand, human serum which when undiluted could neutralize only 1 to 3 infectious doses of virus, when used at a 40 per cent concentration could promote continued serial passage of infected cultures. Rabbit immune serum did not inhibit viral multiplication *in a previously infected culture*, whereas *40 per cent human serum* with one-sixteenth the antibody titer of rabbit antiserum significantly hindered propagation of virus. Both fluids reduced viral cytopathogenicity. Despite these data it is not possible to be certain of the exact role which specific antibodies or other serum inhibitors might play in this phenomenon. It is clear, however, that these factors alone were not sufficient for prolonged maintenance of the viral carrier culture.

The mechanism by which a persistent adenovirus infection of the highly susceptible HeLa cells is initiated and maintained is not entirely clear. From the evidence described, however, the following hypothesis may be deduced: Relatively few cells are infected and under the cultural conditions employed, *viral multiplication is suppressed but not completely inhibited*. The reduction in viral growth appears to be effected by the high concentration of human serum, but not *solely, if at all, by the specific antibodies. It would appear, rather,* that the very active cell metabolism stimulated by the human serum limits viral propagation and perhaps cell injury as well. The virus produced can persist because of its favorable intracellular position and its considerable stability under adverse conditions. The uninfected cells remain susceptible to virus, so that when the cultural conditions are made less favorable, viral multiplication increases, newly produced virus spreads to surrounding cells, and extensive damage of cells occurs.

Just as highly favorable cultural conditions may render cells more resistant to some viral infections and their subsequent effects, so too, poor cell nutrition may make cells less able to support viral infections. An intriguing example of this concept has been presented by MORGAN and his colleagues (MORGAN, 1956; JOHNSON AND MORGAN, 1956; HEGGIE AND MORGAN, 1956; MORGAN AND BADER, 1957; MORGAN, 1957). The experiments do not describe true viral *carrier cultures*

a likeness to the situation which exists in some latent infections in nature.

A viral carrier culture which has certain similarities to the persistent poliomyelitis infection of HeLa cells described, but which also has fundamental differences, is that of a prolonged and continuous adenovirus infection of HeLa cells (GINSBERG AND BOYER, 1956; GINSBERG, 1957a). The demonstration by ROWE and coworkers (1953) that adenoids contain adenoviruses which cannot be detected until they are "unmasked" by *in vitro* culture of the adenoid tissue implied the role these viruses may play as latent agents. The biologic characteristics of adenoviruses (GINSBERG, 1957b) have further suggested the possibility that these viruses may establish an unusual and perhaps stable relationship with infected cells. These factors stimulated the notion that it might be possible to mimic in tissue culture the situation attained by adenoviruses in certain lymphoid tissues in man.

It was possible to establish, with relative ease, a type 3 or 4 adenovirus infection of HeLa cells which did not consume all cells of the culture and which could be maintained and subcultured in series for at least 30 passages with persistence of infectious virus, but without obvious cytopathic changes in the culture. The essential factor appeared to be that active cell metabolism and mitosis be maintained by human serum in a concentration as high as 40 per cent. This fluid was added one hour after infection with a relatively large quantity of virus. It must be emphasized that type-specific neutralizing antibody was present in sera employed unless special precautions were taken in the selection of blood donors. Throughout the prolonged culture series infectious virus was always demonstrable in washed cultured cells, but not in the fluid containing serum. Upon removal of the growth fluid and addition of a maintenance fluid which would not support continuous propagation of cells, cytopathic alterations rapidly appeared and eventually involved all cells in the culture.

Despite the fact that the adenovirus-infected culture had the appearance of an uninfected culture when growth fluid was present, study of fixed and stained preparations demonstrated the presence of a small proportion of cells (0.5 to 5.0 per cent) which had nuclear changes characteristic of infection with the adenovirus employed (BOYER ET AL., 1957). Evidence obtained by clone cultures and by fluorescent antibody studies tended to confirm the evidence that a relatively few cells were infected. Not only were the cultures derived from cell clones not infected, but also, of the 114 clones selected, none

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persistent infections were accomplished in a relatively few strain L cells which resisted the lethal effects induced by western equine encephalomyelitis virus in sister cells. In some cultures thus established, virus was lost after several days to a few weeks. In others, however, virus persisted for as long as 25 months in several serial subcultures. The cultures which showed no detectable cytopathic changes as a result of continued viral multiplication were resistant to subsequent challenge with large quantities of homologous virus. Spontaneous disappearance of virus often occurred, or cultures could be "cured" of viral infection by inclusion of specific immune serum in the fluid media. When virus could no longer be demonstrated in cells or fluid, the cells were once more sensitive to the cytopathic effect of western equine encephalomyelitis virus. It was suggested by CHAMBERS that non-infectious virus protected cells from viral infection and subsequent necrosis by autointerference. It was further postulated that periodic loss of interfering, noninfectious virus from individual cells rendered these cells susceptible to viral infection, and thus supplied some cells in which virus propagated for long periods of time. If spontaneously, or by the presence of antiserum, all cells lost interfering materials, a completely susceptible population of cells appeared. This mechanism is similar to the one suggested by DEINHARDT ET AL. for infections produced in resistant cell lines (1957; BERGS, 1958). The hypothesis proposed is not an unreasonable one to explain certain types of persistent viral infections *in vitro* or even *in vivo*. Additional evidence, however, concerning such points as the number of infected cells, actual presence of viral material in resistant cells, number of resistant cells, and ability of resistant cells to adsorb virus would be desirable to support the proposed contention.

Summing-up

From a number of laboratories within the past 2 to 3 years have come reports of cells being grown *in vitro* through many generations accompanied by a concomitant viral multiplication in some of the cultured cells. Most of the persistent viral infections described are still under active investigation, and many of the experimental results which appear necessary to permit accurate interpretation of data are not yet attained. Thus, an appraisal of these studies places the review-

because each is confined to a single culture lasting from 7 to 28 days. Nevertheless, the data obtained may have profound significance for the subject at hand and therefore warrant discussion.

Chick embryo tissues and strain L cells are highly susceptible to infection by psittacosis virus. Under conditions of nutritional deficiency, however, it was discovered that the cells could be infected but the virus was not detectable until proper nutrients were added to the culture fluid. Viral multiplication then commenced within 24 hours and progressed so that virus attained high titers. Virus could be maintained in a noninfectious state in "starved" cells for as long as 15 days, after which viral synthesis could be stimulated by the proper nutrients. During this lengthy latent period virus could not be detected and was considered to be in a noninfectious state, although quantitative considerations have not been eliminated as the reason virus was not measurable. To obtain virus in relatively high titer it was only necessary to add water-soluble vitamins, glucose and certain amino acids. The amino acids essential for psittacosis virus propagation in L cells were: threonine, methionine, isoleucine, phenylalanine, tryptophane, leucine, valine, cystine (or cysteine) and tyrosine. For chick embryo tissues phenylalanine and tryptophane were necessary for viral multiplication. It is clear from these experiments that the nutritional factors required to be added to cells to permit them to support viral synthesis were relatively simple. It must be realized, however, that these studies shed no light as to the stores of metabolically active materials and enzymes which persist in cells even after prolonged starvation. Thus, although the experiments described present a model whereby in culture virus can persist in a state undetectable by usual methods, but remains capable of propagation, the data do not necessarily indicate the building blocks required for psittacosis viral synthesis. Indeed, it cannot be inferred that the situation described simulates the condition of latency for psittacosis virus in birds or man, but rather this interesting investigation indicates one mechanism by which it is possible for a virus to persist in host cells in an inapparent state. The experimental situation developed by MORGAN and colleagues with psittacosis virus in nutritionally deficient tissue culture cells resembles the inapparent or avirulent infection with lymphocytic choriomeningitis virus in folic acid deficient mice by HAAS and his coworkers (1956, 1957).

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Chick embryo tissues and strain L cells are highly susceptible to infection by psittacosis virus. Under conditions of nutritional deficiency, however, it was discovered that the cells could be infected but the virus was not detectable until proper nutrients were added to the culture fluid. Viral multiplication then commenced within 24 hours and progressed so that virus attained high titers. Virus could be maintained in a noninfectious state in "starved" cells for as long as 15 days, after which viral synthesis could be stimulated by the proper nutrients. During this lengthy latent period virus could not be detected and was considered to be in a noninfectious state, although quantitative considerations have not been eliminated as the reason virus was not measurable. To obtain virus in relatively high titer it was only necessary to add water-soluble vitamins, glucose and certain amino acids. The amino acids essential for psittacosis virus propagation in L cells were: threonine, methionine, isoleucine, phenylalanine, tryptophane, leucine, valine, cystine (or cysteine) and tyrosine. For chick embryo tissues phenylalanine and tryptophane were necessary for viral multiplication. It is clear from these experiments that the nutritional factors required to be added to cells to permit them to support viral synthesis were relatively simple. It must be realized, however, that these studies shed no light as to the stores of metabolically active materials and enzymes which persist in cells even after prolonged starvation. Thus, although the experiments described present a model whereby in culture virus can persist in a state undetectable by usual methods, but remains capable of propagation, the data do not necessarily indicate the building blocks required for psittacosis viral synthesis. Indeed, it cannot be inferred that the situation described simulates the condition of latency for psittacosis virus in birds or man, but rather this interesting investigation indicates one mechanism by which it is possible for a virus to persist in host cells in an inapparent state. The experimental situation developed by MORGAN and colleagues with psittacosis virus in nutritionally deficient tissue culture cells resembles the inapparent or avirulent infection with lymphocytic choriomeningitis virus in folic acid deficient mice by HAAS and his coworkers (1956, 1957).

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Table 1 Summary of possible mechanisms for maintenance of viral carrier states in tissue culture

Virus-Cell System		Mechanism		Reference
Virus	Host Cell	Primary	Secondary	
NDV, MV, 6-6	MCN, Lung-To	Resistant cells	Interference phenomenon	Deinhardt, et al (1957) Bergs (1958)
Coxsackie A9	HeLa	Resistant cells	Active cell growth and metabolism	Takemoto and Habel (1958)
NDV	HeLa	Resistant cells		Puck and Cieciura (1957)
Eastern equine	Rat fibroblasts	Resistant cells		Bang and Gey (1952)
Psittacosis	Chick embryo L cells	Cell starvation		Morgan (1956)
Western equine	L cells	Interference phenomenon		Chambers (1957)
Poliomyelitis	HeLa	Antibody	Selected resistant cells	Ackermann and Kurtz (1955)
Adenovirus	HeLa	Active cell growth and metabolism	Antibody or other serum inhibitors	Ginsberg and Boyer (1956)

phenomena by comparing them with viral carrier states or latent infections in the intact animal It would not be inconceivable that mechanisms similar to those postulated to be the basis of the viral carrier cultures might be operative in persistent viral infections in nature. The limiting factor in such comparisons, however, is that the means by which the viral carrier state or latent infection is attained in animals cannot be investigated as readily as the *in vitro* models.

er in a less enviable position than that assumed by a contemporary historian who describes and interprets recent but already completed events. Despite these obvious difficulties, a *summing-up of information* available and discussion of its possible significance may be of value to those investigators concerned with these or similar problems.

In general, the viral carrier cultures which have been described were discussed in 3 main groups. The persistent infections were categorized as those established and maintained in: 1) cell cultures in which the majority of cells were primarily resistant to infection with the agents employed; 2) resistant cells which were selected by pressure of viral action from a population of cells, the majority of which were killed by the viral infection; and 3) cells which were highly susceptible to viral infection. In the persistent viral infections described, none could be formally compared to the situation of lysogenic bacteria. In most instances the data clearly indicate that the number of cells supporting viral infection was only a small proportion of the entire population. In some, although relatively few cells contained infectious virus, it was suggested that most of those remaining were made resistant by noninfectious viral material. The model of HeLa cells infected with Newcastle disease virus appeared to be one in which the majority of cells contained infectious virus; further experiments, however, are necessary to establish this important point (PUCK AND CIECIURA, 1957).

Despite the variety of viruses employed, the divergence of cell types used, and the differences in the reactions of the viruses with host cells, only a few mechanisms appear to be involved in these viral carrier states. They are summarized in table I. In some of the virus-cell systems described it is possible that more than one mechanism was operative. One basic and essential factor which appears to be germane to all of the viral carrier states described, however, is that the majority of cells in culture were resistant to the injurious effects of the virus. This state of relative insusceptibility was attained by several different means: 1) natural or selected resistance; 2) nutritional starvation of the host cells; 3) presence of noninfectious viral material initiating interference with infection by active particles; 4) specific-antibody or other serum inhibitors of viral infection; and 5) maintenance of cells in a state of high metabolic vigor and active cell propagation.

Because the viral carrier state in tissue culture is a pure *in vitro* model, it would seem profitable to evaluate the significance of these

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phenomena by comparing them with viral carrier states or latent infections in the intact animal. It would not be inconceivable that mechanisms similar to those postulated to be the basis of the viral carrier cultures might be operative in persistent viral infections in nature. The limiting factor in such comparisons, however, is that the means by which the viral carrier state or latent infection is attained in animals cannot be investigated as readily as the *in vitro* models.

There are several examples of persistent viral infections in which the animal is infected either *in utero* or very early in life, and thereafter viral infection continues and infectious virus can be readily demonstrated although the animal apparently remains healthy. Two examples of this situation which have been thoroughly investigated and well described are the chronic infections of mice by lymphocytic choriomeningitis virus (TRAUB, 1936), and THEILER'S virus (THEILER, 1937; THEILER, 1941). The exact mechanisms by which these infections are maintained have not been elucidated, but the following hypothesis would explain the known facts; Infectious virus could persist by multiplying in a relatively few susceptible cells in tissues in which the majority of cells are insusceptible, or by propagating in cells which support viral synthesis but which are not injured by the viral infection. Spread of infectious virus to susceptible cells then is limited by specific neutralizing antibody, or cells may be made partially resistant by noninfectious viral material which interferes with subsequent infections. The hypothesis proposed as the *modus operandi* by which persistent infections in mice with THEILER'S or lymphocytic choriomeningitis virus are maintained is based upon mechanisms which are probably operative in certain of the tissue culture models described.

Latent or recurrent infections such as infection with herpes simplex virus in man (ANDERSON AND HAMILTON, 1949) or pneumonia virus in mice (PVM) (HORSFALL AND HAHN, 1940, HORSFALL, 1958) exemplify the situation in which virus is not demonstrable after primary infection, and disease does not occur until provocative factors initiate active viral multiplication, cell damage and a localized clinical infection. Certain of the mechanisms involved in the viral carrier states in tissue culture could be similar to those which pertain in latent infections of man or animals. In herpes simplex viral infections or other latent infections there is no evidence as to the state of the virus, infectious or noninfectious, during the silent periods of the infectious process. With PVM, however, virus is not detectable while the animal is healthy. The inability to detect virus may be merely a quantitative matter, as suggested by BEARD for Shope papilloma virus (BEARD, 1956), or may be the result of the interaction of virus and neutralizing antibody when materials are obtained for virus isolation. Thus, in the examples under consideration, virus may propagate in relatively few cells whose damage may not be detectable, and surrounding cells are not affected because they have assumed a resistant state. The relative

insusceptibility of surrounding cells may be a consequence of specific antibody in interstial fluid, metabolic or nutritional factors, or of noninfectious viral materials invoking the interference phenomenon. Provocation to active infection may be accomplished by: 1) Reduction of antibody in the fluid bathing the cells either as a result of diminished total antibody or decreased circulation; 2) reduction of the metabolic activity of the surrounding cells and diminution of their vigorous cell growth thereby decreasing their resistance to viral infection; 3) increase in susceptibility of the cells by alteration in their nutritional status; or 4) reduction of the quantity of interfering noninfectious viral material Any of the above means to increase the susceptibility of the majority of cells permits virus to spread from the few affected cells and thus initiate an active infection.

One cannot disregard the biologic properties of the agents involved in such situations for they must be able to persist for long periods in a relatively few cells. Adenoviruses appear particularly well suited to this task. they are quite stable to adverse conditions of pH and temperature (GINSBERG, 1956): they multiply in nuclei of cells (KJELLEN ET AL., 1955; MORGAN ET AL., 1956; HARFORD ET AL., 1956) and only a small proportion of the virus synthesized emerges from cells to be exposed to inactivating substances such as antibodies (GINSBERG, 1958); and perhaps most important, they apparently do not lyse and perhaps do not even kill the cells in which they multiply (GINSBERG, 1957).

It is apparent from this discussion that the actual mechanisms by which viral carrier states or latent infections are maintained in the whole animal are not clearly defined. The various models of the viral carrier state in tissue culture which have been described suggest that the basic mechanism, relative cell resistance, which apparently is operative in all of the models, may be attained by several means. Consideration of similar carrier states in animals permits the use of this notion of relative cell resistance in the majority of cells to explain the chronic, inapparent, or latent infections. Thus, although there are still many questions to be answered and a number of details to be clarified, the development of a variety of viral carrier states in tissue culture has suggested mechanisms for similar infections in nature, and approaches for elucidation of these problems.

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ADVANCES IN THE STUDY OF THE ENTEROVIRUSES

By JOSEPH L. MELNICK

Because of their many similarities, the poliomyelitis, Coxsackie, and ECHO groups of viruses have recently been brought together as a single family of *enteroviruses* (Committee on the Enteroviruses, 1957). The number of newly recognized viruses of the human intestinal tract has grown rapidly in the last few years, at the present writing 51 distinct viruses are known (Table I), and many more Coxsackie and ECHO strains distinct from the established types remain to be classified. Other viruses, particularly those of the adenovirus group, occasionally may be found in the intestinal tract, but they are

Table I. Number of Enteroviruses Presently Classified

Virus Group	Number
Polioviruses	3 types
Coxsackie Viruses	
Group A	19 types
Group B	5 types
ECHO Viruses	24 types

easily recognized as being different from the true enteroviruses. The virus of infectious hepatitis might well be included when more is learned about its properties. In this review, only *selected* aspects of current research in the enterovirus field will be considered.

Polioviruses

Not long ago poliovirus was considered to be strictly neurotropic, but now it is universally held that invasion of the nervous system is the unusual event during an infection with this agent In fact, many of the strains isolated from normal individuals have such a low affinity for primate neurones that their very existence would not have come to light if it were not for having *in vitro* tissue culture systems as indicators of their presence. Thus attenuated strains of all three polioviruses have been isolated directly from inapparent human carriers (RAMOS-ALVAREZ AND SABIN, 1954; HONIG ET AL., 1956; FOX, 1957). In addition, attenuated strains have been obtained by manipulation of virulent strains in the laboratory (SABIN ET AL., 1954; MELNICK, 1954, LI AND SCHAEFFER, 1955; KOPROWSKI, 1955, LEPINE, 1957). Such strains are being considered as possible live virus vaccines, to be given orally either alone or after vaccination with killed virus The reader is referred to the recent publication, "*Cellular Biology, Nucleic Acids, and Viruses*" (New York Academy of Sciences Special Publications, Volume V, 1957), where this subject is dealt with by several investigators: SABIN, PAUL, KOPROWSKI, LEPINE, FOX, STUART-HARRIS, DICK, and SHOPE.

Enteric infection after Salk vaccination. When the Salk vaccine was first introduced, the question was raised as to whether it might not interfere with the natural immunity which follows inapparent infections. If such were the case, then paralytic poliomyelitis might only be postponed for some years, perhaps to a period when the disease would be more dangerous than that of childhood. Studies have been undertaken in order to determine whether persons immunized by Salk vaccine are as likely as those not vaccinated to become intestinal carriers of poliovirus upon subsequent exposure. In studies in which attenuated polioviruses were fed to human subjects (SABIN, 1957; PAUL ET AL., 1957), intestinal infection with attenuated viruses occurred as readily in persons exhibiting antibodies acquired from previous Salk vaccine as in persons without antibodies. Similar observations were made on vaccinated children

exposed to natural contagion from healthy carriers (GELFAND ET AL., 1957) In a study of the fecal excretion of poliovirus among household contacts of *clinical* cases of poliomyelitis (DAVIS ET AL., 1958), again, no significant difference in virus excretion was found between children who had received vaccine, and those who had not been previously vaccinated. No effect could be demonstrated upon the duration of virus excretion nor upon the amount of virus present in the feces. The over-all evidence to date suggests that infection in the first year after the vaccine is given results in a boost in immunity. The consequences of infection at a later period, when vaccine-induced antibody has disappeared, remains to be determined

From the results of these studies, it would appear that the amount of contagion and the incidence of poliomyelitis infection, in contrast to paralytic disease, may not be significantly reduced as a result of Salk vaccination. Thus, the rate of infection within the community should not differ from that observed before the introduction of vaccination, and the opportunity should remain for active immunity resulting from infection to be superimposed upon that acquired from vaccination. This may be a more desirable situation than if the virus were eventually to be eliminated, since if this were to occur, the population would then be entirely dependent for protection upon artificial immunization. Consequently, if vaccination were allowed to lapse in adult life with a resulting fall in antibody levels, the stage might then be set for a serious epidemic, when the poliovirus was reintroduced into the population—as it would almost certainly be, in view of its prevalence throughout much of the world

Genetic stability of attenuated poliovirus strains Perhaps the most serious question to be answered in the studies with live virus vaccines is the stability of the virus in the community, after it has been excreted by the person who swallows the attenuated form of the agent (SABIN, 1957, HORSTMANN ET AL., 1957; KOPROWSKI ET AL., 1957; DICK AND DANE, 1957). There is ample evidence to show that infection spreads readily to those in contact with children who excrete the virus (PAUL ET AL., 1957) Attenuated viruses when fed in high doses produce infections even in individuals who have antibody—although to a much lesser extent in those with naturally induced antibodies as against the almost 100 per cent infections in those with Salk vaccine-induced antibody. It is perhaps even more important to determine the stability of attenuation after virus multiplies in persons with antibodies, as it is in those without antibodies

Fig 1 Plaque formation of virulent (MAHONEY) poliovirus at different concentrations of sodium bicarbonate as indicated Each culture received the same dose of virus Approximately the same number of plaques is present on the 4th day after seeding, when the photographs were taken

MAHONEY 0 1 gm%

MAHONEY 0 2 gm%

MAHONEY 0 5 gm%

MAHONEY 0.9 gm%

Y-SK 0 1 gm%

Y-SK 0 2 gm%

Y-SK 0 5 gm%

Y-SK 0 9 gm%

Fig 2 Plaque formation of an attenuated (Y-SK) poliovirus Conditions were the same as those indicated for Figure 1. Virus growth under agar was delayed as the concentration of bicarbonate was decreased No plaques developed at the lowest concentration of bicarbonate.

Because it is so laborious and expensive to determine the neuro-irulence of poliovirus by inoculation into monkeys, an *in vitro* marker of this character is sorely needed. An important lead in this irection was made by VOGT ET AL. (1957), who recognized a genetic ifference between neurovirulent strains and certain attenuated trains of poliovirus, namely the lessened capacity of attenuated trains to produce plaques under a slightly acid agar overlay. Be-ause they are unable to produce plaques, or produce delayed pla-ues, under the acid overlay, they are designated *d*, in contrast to he wild type, d^+. In bottle cultures also the concentration of bicar-onate in agar overlay affects the plaque formation of attenuated trains of poliovirus (HSIUNG AND MELNICK, 1958b) In contrast to irulent d^+ strains (Figure 1) which grow equally well under agar ontaining low (0.11 gm per cent) or high (0.45 gm per cent) con-entrations of sodium bicarbonate, attenuated *d* strains grow poorly nd form relatively few plaques at the low bicarbonate concentration Figure 2). Adjusting the initial pH of the agar by addition of NaOH r CO_2 did not influence plaque development of *d* strains



Fig 3 Titration of poliovirus recovered from a child fed attenuated Type 1 LSc strain Amount of bicarbonate in overlay 0 45 gm per cent Inoculum 0.1 ml of first passage tissue culture fluid, at concentration of 10^{-4} (left), 10^{-5} (middle), and 10^{-6} (right). Photographs taken 4 days after seeding



Fig 4 Titration of same material as shown in Figure 3, but at bicarbonate concentration of 0 1 gm per cent No plaques present at virus final inocula of 10^{-2} through 10^{-5}, representing challenge concentrations of over 12000 PFU (when measured at the higher bicarbonate concentration)

In liquid medium the concentration of bicarbonate in the range of 0.11 to 0.45 gm per cent did not affect the attachment of virus to the cell nor the pattern of the growth curves either of attenuated strains or of virulent ones. This lends support to the conclusion of VOGT AND DULBECCO that under slightly acid agar, the susceptibility of the cells to attenuated strains becomes reduced only after a period equivalent to a single growth cycle of the virus.

HSIUNG AND MELNICK (1958b) have analyzed a large number of poliovirus strains for the *d* character, to determine whether this might provide an *in vitro* method for differentiating virulent from attenuated strains of poliovirus as they occur in nature, and in stools of persons fed attenuated strains of poliovirus (Figures 3 and 4). First it was established that strains passaged in monkey spinal cord retained the neuropathogenic character of the parent line. When the original inoculum was a virulent virus, the strains on further passage in tissue culture yielded d^+ viruses. Similarly, when the parent virus was attenuated, the tissue culture progeny were of the

d type, *even after multiplication in the nervous system.* However, two exceptions were encountered where d^+ progeny were produced from *d* parents, but the d^+ progeny still behaved as attenuated strains, in that they were almost free of intracerebral neuropathogenicity for the monkey. SABIN (1957) has also observed that the d^+ character may at times be associated with polioviruses of the attenuated variety.

A number of natural and newly isolated strains, consisting of virus particles as present in human stools and in early tissue culture progeny, were also analyzed (HSIUNG AND MELNICK, 1958b). In general, epidemiologically paralytogenic strains had a high efficiency of plating in low bicarbonate medium, the property of a d^+ virus. Strains which were epidemiologically mild, or which were recovered from normal children in inter-epidemic periods, fell into both the *d* and d^+ categories. Thirty-two isolates were tested, both for the *d* character and for neurovirulence in monkeys. The d^+ strains proved to be usually paralytogenic for the monkey, but sometimes pathogenicity was demonstrable only by histological examination of the spinal cord. In contrast, the *d* strains in this group failed to produce any apparent disease in the test monkeys, however, lesions of a limited character were induced in the spinal cords of a number of the animals.

Testing for the *d* character of a poliovirus (and for its mutation to d^+) is a convenient method for determining the genetic stability of attenuated, live virus vaccines. When an attenuated *d* strain was fed to human beings, this was followed usually by the excretion of the parent *d* virus, but in certain individuals by the excretion of d^+ viruses (HORSTMANN ET AL., 1957; HSIUNG AND MELNICK, 1958b). However, the d^+ mutants were still of low neuropathogenicity for the monkey.

The recent work on the *d* character of poliovirus suggests that a mutation in the *d* direction is associated with attenuation, but that attenuation may also result from other genetic changes in the virus. The results on finding attenuated d^+ variants in the progeny of *d* strains (HSIUNG AND MELNICK, 1958b; SABIN, 1957; DULBECCO, 1957) supports the view that any reverse mutation to virulence is the result of a series of step-wise alterations within the genetic constitution of the virus.

KANDA AND MELNICK (1958) have discovered that virulent and attenuated polioviruses may also be differentiated *in vitro* by their growth characteristics on a monkey kidney continuous cell line. The growth patterns of the three strains of poliovirus used in the pre-



Fig 4 Titration of same material as shown in Figure 3, but at bicarbonate concentration of 0.1 gm per cent. No plaques present at virus final inocula of 10^{-2} through 10^{-5}, representing challenge concentrations of over 12000 PFU (when measured at the higher bicarbonate concentration)

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naked eye at autopsy. Brain involvement may be characterized by apparent softening of the tissue. Although striated muscles are attacked by group B viruses, the myositis is focal and limited. The lesions most useful in characterizing the group B viruses are degeneration of the brown fat, the intrascapular fat pads, encephalomalacia, and—to a lesser degree—hepatitis, myocarditis, and necrosis of the acinar tissues of the pancreas.

The pancreatic lesion has been used by HOWES (1954) as a means of titrating group B strains. Mice in the titration series are inoculated intraperitoneally with trypan blue, and four hours later, when the animals are sacrificed, the pancreatic lesions have become stained and visible to the naked eye.

Mouse pathogenicity has been regarded as a cardinal characteristic of the Coxsackie viruses. However, recent studies of naturally-occurring strains have shown that the situation is, in some regards, similar to that which occurs with the polioviruses, in that a number of strains, avirulent for the mouse, have been isolated in tissue culture. Thus, at least 35 strains (belonging to A-9 and to B-1 to B-5) have been observed (HABEL et al., 1957; DAVIS AND MELNICK, 1958) which could be isolated in tissue culture, but all of which gave negative tests in suckling mice. Even after passage in tissue culture, a number of these strains still remained avirulent for suckling mice. It must be emphasized that there are also Coxsackie viruses which have been isolated in suckling mice but which have failed to grow in tissue culture, even though other strains of the same type are cytopathogenic for monkey kidney.

One strain of Coxsackie A-14 has been adapted to adult mice, and produces poliomyelitis-like lesions in them (DALLDORF, 1957). The adapted virus retains its pathogenicity for suckling mice, in which it produces only myositis. In monkeys, the adult-mouse adapted strain produces poliomyelitis-like lesions, even though these are not extensive enough to result in overt paralysis. Coxsackie A-7 strains have also been found to induce such CNS lesions in monkeys (DALLDORF, 1957; JOHNSSON AND LUNDMARK, 1957; HABEL AND LOOMIS, 1957; CHUMAKOV ET AL., 1956; HORSTMANN AND MANUELIDIS, 1957). It is of interest that CHUMAKOV and his associates isolated Coxsackie A-7 from patients with poliomyelitis-like paralysis, and that the strain isolated by them produced paralysis in monkeys.

Antigenic variants among the Coxsackie viruses. Recent results in two laboratories (SABIN AND WIGAND, 1957; MELNICK AND KANDA,

paration of vaccine (Mahoney, MEF, and Saukett), and those of three attenuated strains (LSc, Y-SK, and Leon), were studied in primary monkey kidney (MK) and in a continuous stable cell line derived from this source (MS). In cultures in fluid medium, all six strains yielded approximately the same titers in MK and in HeLa cultures; however, in MS cultures, the LSc, Y-SK, Leon, and Saukett strains yielded titers as low as 0 01 per cent of the values obtained in MK cultures. When assayed by the plaque method, all 6 strains on MK monolayers yielded values between $10^{7.3}$ and $10^{8.4}$ PFU per ml, and the plaque size was between 13 and 18 mm by the sixth day. Again, there were marked differences on the MS monolayer cultures: highly virulent Mahoney and MEF produced plaques of 3 to 5 mm diameter, while the remaining 4 strains yielded much lower titers and plaques less than 1 mm in diameter. Thus in addition to the restricted growth of most attenuated polioviruses at low bicarbonate concentrations, described above, another marker for attenuation seems to be their restricted growth on MS cells. The poor growth of the attenuated strains, and of Saukett, is in agreement with their low degree of neurovirulence for monkeys.

DUBES AND WENNER (1957) have been able to select poliovirus variants of decreased neurovirulence by isolating progeny which grow in cell cultures maintained as low as 23° C. In their experiments, terminal-dilution end point passages in cell cultures resulted in the emergence of viruses of reduced neurovirulence. Further reductions in virulence were obtained by sequential adaptation of the viruses to multiply at 30° and then at 23° C. These investigators believe that the relative avirulence of the cold-adapted virus is due to its reduced capacity to multiply at 37°.

Coxsackie Viruses

The Coxsackie viruses have been separated into groups A (19 types) and B (5 types), depending upon the disease produced in infant mice and the attendant character and distribution of the lesions in these animals (DALLDORF, MELNICK, AND CURNEN, 1958). Members of group A produce a prostrating paralysis, and death soon follows. In such animals there is a generalized and widespread myositis, and tissues other than striated muscle are spared. Group B viruses produce tremors, spasticity, and a spastic paralysis. Changes in the intrascapular fat pads may occur which can be seen with the

cytopathogenic agents—originally called "orphan" viruses or human enteric viruses but more recently ECHO (enteric cytopathogenic human orphan) viruses—were isolated from the human intestinal tract. Preliminary studies indicated that different antigenic types existed. This has been borne out by subsequent work which has differentiated 24 antigenically distinct types, listed in Table III. None of the prototype viruses produce disease in laboratory animals, including infant mice.

The Committee on the Enteroviruses (1957) has been set up to assist in the characterization and classification of new prototypes of any member of the human enterovirus group. This Committee (1955) had defined the ECHO viruses as a group of agents sharing the following properties: (1) They are cytopathogenic for monkey and human

Table III. List of Antigenically Distinct ECHO Viruses

Type	Prototype strain	Geographic origin	Illness in person yielding prototype virus	Investigator
1	Farouk	Egypt	none	Melnick
2	Cornelis	Connecticut	Aseptic meningitis	Melnick
3	Morrisey	Connecticut	Aseptic meningitis	Melnick
4	Pesascek	Connecticut	Aseptic meningitis	Melnick
5	Noyce	Maine	Aseptic meningitis	Melnick
6	D'Amori	Rhode Island	Aseptic meningitis	Melnick
7	Wallace	Ohio	none	Ramos-Alvarez, Sabin
8	Bryson	Ohio	none	Ramos-Alvarez, Sabin
9	Hill	Ohio	none	Ramos-Alvarez, Sabin
10	Lang	Ohio	none	Ramos-Alvarez, Sabin
11	Gregory	Ohio	none	Ramos-Alvarez, Sabin
12	Travis	Philippine Islands	none	Hammon, Ludwig
13	Hamphill	Philippine Islands	none	Hammon, Ludwig
14	Tow	Rhode Island	Aseptic meningitis	Melnick
15	CH 96-51	West Virginia	none	Ormsbee, Melnick
16	Harrington	Massachusetts	Aseptic meningitis	Kibrick, Enders
17	CHHE-29	Mexico City	none	Ramos-Alvarez, Sabin
18	Metcalf	Ohio	Diarrhea	Ramos-Alvarez, Sabin
19	Burke	Ohio	Diarrhea	Ramos-Alvarez, Sabin
20	JV-1	Washington, D C	Fever	Rosen
21	Farina	Massachusetts	Aseptic meningitis	Enders, Kibrick
22	Harris	Ohio	Diarrhea	Sabin et al.
23	Williamson	Ohio	Diarrhea	Sabin et al
24	De Camp	Ohio	Diarrhea	Sabin et al

Table II Antigenic Variation Within Coxsackie B Types*

Type	Strain	Serum Titers vs 100–32 TCD_{50} of Virus		
		B-2	B-3	B-4
B-2	Ohio-1	630–1100	0	0
	9 new strains	6–75	0	0
B-3	Nancy	0	150–1300	0
	9 new strains	0	<2–210	0
B-4	Powers	0	0	125–400
	9 new strains	0	0	14–160

* Data taken from SABIN AND WIGAND (1957) and MELNICK AND KANDA (1957)
0 Indicates less than 10
Rabbits were immunized with the reference strains (Ohio-1, Nancy and Powers)
The neutralization titers given are ranges of serum titers observed in both laboratories.

1957) have shown that Coxsackie B strains recently isolated in tissue culture do not cross completely with the reference strains. Some results are shown in Table II. Some strains of the same type are more difficult to neutralize than others, and also some strains are antigenically more complex. Both laboratories noted, however, that even the strains against which the reference antisera yielded the lowest titers, or negative titers, at the end of an 8-day neutralization test *in tissue culture, nevertheless did exhibit some neutralization and* higher titers at earlier readings (after 3 to 5 days). This type of breakthrough, which occurs in tube cultures, may be overcome if plaque neutralization tests are done (MELNICK, 1957; DAVIS AND MELNICK, 1958). On passage of mouse-adapted Coxsackie B viruses through tissue cultures, variants may appear which are not identical antigenically with the parent strain. Typing sera for the Coxsackie viruses have recently been prepared under the direction of the Committee on the Enteroviruses of The National Foundation for Infantile Paralysis (1957), and are now available to virus laboratories.

ECHO Viruses

Soon after tissue culture techniques were introduced into laboratories concerned with the isolation of viruses, large numbers of new

cytopathogenic agents—originally called "orphan" viruses or human enteric viruses but more recently ECHO (enteric cytopathogenic human orphan) viruses—were isolated from the human intestinal tract. Preliminary studies indicated that different antigenic types existed. This has been borne out by subsequent work which has differentiated 24 antigenically distinct types, listed in Table III. None of the prototype viruses produce disease in laboratory animals, including infant mice.

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cells in culture. Monkey kidney cells are more susceptible to the viruses as they exist in human hosts than are HeLa cells. (2) They are not neutralized by pools of the three types of poliomyelitis antiserum. (3) They are not neutralized by antisera for Coxsackie viruses, and they fail to induce disease in infant mice. (4) They are not related to other groups of viruses recovered from the alimentary tract (throat or intestine), such as *herpes simplex, mumps, measles, varicella*, and adenoviruses. (5) They are neutralized by human gamma globulin, and by individual human sera, indicating that they infect human beings.

The ECHO viruses were separated from the Coxsackie viruses, because only the latter were pathogenic for newborn mice. Classification problems have arisen as a result of the findings that the 1956 European and subsequent strains of ECHO-9 (but not the prototype and other strains) produce paralysis in mice (JOHNSSON, 1957; QUERSIN-THIRY ET AL., 1957; BOISSARD ET AL., 1957; TYRELL ET AL., 1957; GODTFREDSEN AND VON MAGNUS, 1957; MCLEAN AND MELNICK, 1957; SABIN ET AL., 1958). Mice develop a widespread myositis like that produced by Coxsackie A viruses. However, unlike the Coxsackie viruses, the original material collected from the ECHO-9 *patients failed to produce disease in newborn mice. Passage in tissue* culture was required in order to select out mouse pathogenic virus particles, but even after tissue culture passages, only a certain proportion of the strains proved mouse pathogenic. EGGERS AND SABIN (1958) have studied quantitatively the variation among ECHO-9 strains. They found that some fail to multiply in mice regardless of the dose inoculated, while others multiply even when as little as 3 TCD_{50} of virus are injected, with larger doses necessary to cause paralysis. That the mouse pathogenic variant was identical to the parent virus grown in tissue culture was shown by neutralization tests carried out in newborn mice as well as in tissue culture, with both lines of virus (MELNICK, 1957b).

Some ECHO-10 strains also produce a disease in mice similar in many respects to that of Coxsackie B (DALLDORF, 1957; SABIN, 1957) However, this virus is so

Reference antisera. In the course of the establishment of a prototype strain, reference prototype sera have to be prepared and crossed

with the previously known types. In order to expedite work in this field, large pools of antisera have now been prepared in rabbits and in monkeys, under the sponsorship of The National Foundation for Infantile Paralysis, acting through the Committee on the Enteroviruses. Standardization of these sera has recently been described by the Committee (1957).

Reference antisera are now available. All have high titer except in the case of type 4 serum. However, if this antiserum is tested by the more sensitive plaque reduction method, then its titer is satisfactory (1:2000). Others (CHIN ET AL., 1957; ITOH AND MELNICK, 1957) have also commented on the difficulty of detecting neutralizing antibodies with type 4 virus in tube neutralization tests. In one series of experiments, it was only by the plaque reduction method that it could be demonstrated that chimpanzees responded to exposure to the virus by developing neutralizing antibodies.

Within certain ECHO types there exist strains which are not uniform in antigenicity (MELNICK, 1956; 1957; KARZON, 1957, SABIN, 1957; HAMMON, 1957; Committee on the Enteroviruses, 1957) Such broad antigenic strains have been called prime strains. Although antiserum to a prime strain neutralizes other strains within its type, the prime strain itself may be poorly neutralized by antisera prepared against other strains of the same type. To date, prime strains of type 6 have been recovered most frequently. KARZON (1957) suggests that during the course of a type 6 epidemic, variants of the virus appear with modified antigenicity. Noteworthy are the 6-prime strains that produced typical ECHO plaques (HSIUNG AND MELNICK, 1957a), whereas the 6 prototype strain has not Naturally occurring strains belonging to types 5, 7, 9, 10, 11, and 13 are known which also exhibit only partial crossing with their respective prototypes

Cross relationships between ECHO types. The crossing which has existed in certain passages of types 1 and 13 is now believed to be due to a mixture of the two viruses (HAMMON, 1957; Committee on the Enteroviruses, 1957). However, the crossing found between types 1 and 8 cannot be explained on this basis because plaque-purified type 8 virus passed in the presence of type 1 antiserum still manifests the same partial cross-neutralization. Some illustrative data of BENYESH AND MELNICK (1957) are shown in Table IV. The crossings which have been found to exist in neutralization tests are also manifested in cross complement fixation tests (ARCHETTI ET AL., 1957; Committee on the Enteroviruses, 1957).

Table IV. Cross Reactions Between Plaque-Purified Prototype ECHO Viruses 1 and 8 And A Newly Isolated (Hall) Strain*

(90% Plaque Reduction Endpoint)

Virus	Virus Dose (PFU)	Serum Titers			
		ECHO-1 (Monkey)	ECHO-8 (Rabbit)	ECHO-1 (Rabbit)	ECHO-8 (Rabbit)
ECHO-1 (Farouk)	650	31250	50	250	10
ECHO-8 (Bryson)	500	1250	6250	10	1250
Hall	850	6250	6250	25	600
Neutralization Ratio = Titer against heterologous virus / Titer against homologous virus					
ECHO-1		1.00	0 01	1 00	0 01
ECHO-8		0 04	1 00	0 04	1 00
Hall		0.20	1 00	0 10	0 50

* From unpublished experiments of BENYESH AND MELNICK, 1957. The Hall strain was kindly provided by Dr LEON ROSEN.

A report (KRECH, 1957) on crossing between ECHO-9 and herpes simplex virus could not be confirmed (MELNICK, BENYESH, AND WARREN, 1957). When cross neutralization tests were carried out with falling dilutions of the reference monkey ECHO-9 antiserum and a guinea pig herpes antiserum against fixed doses of each virus, only homologous neutralization was achieved. The herpes virus tests were carried out in human amnion cells, the ECHO-9 tests in monkey kidney cultures. Cross complement fixation tests also failed to indicate any crossing between the viruses.

Properties of the Enteroviruses in Tissue Culture*

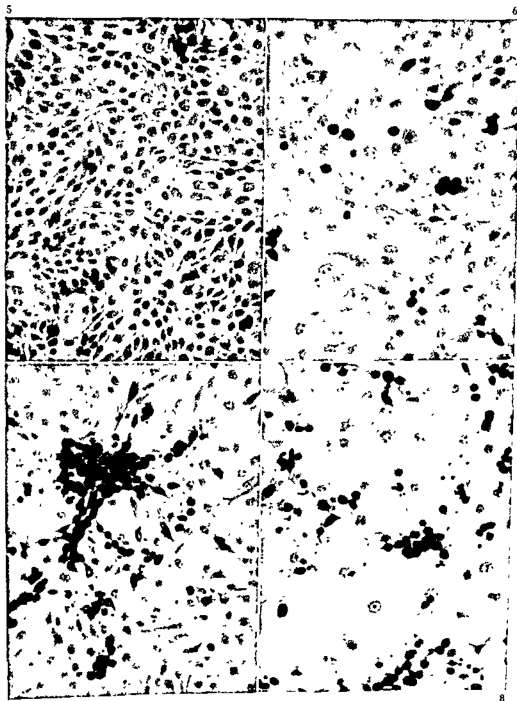
Polioviruses. It was the growth of poliovirus in tissue culture (ENDERS ET AL., 1949) that stimulated much of the current activity in virology and cytology. The recently published Proceedings of the

* An extensive review of tissue culture methods used for the enteroviruses may be found in the chapter by J. L. MELNICK in "Diagnostic Procedures for Virus and Rickettsial Diseases", 2nd Ed. (Amer. Publ Hlth. Association, New York 1956)

Decennial Review Conference on Tissue Culture (1957) and the volume, Cellular Biology, Nucleic Acids, and Viruses (1957) bear adequate testimony to this fact. In this latter volume, PARKER ET AL. (1957) discussed alterations in cells carried in serial culture. As is well known, monkey kidney cells are highly susceptible to poliovirus, but certain types of altered cells which arise from such cultures fail to support the multiplication of the virus. The cell changes in such cultures seem permanent, for the poliovirus-resistant cells continue to multiply indefinitely even in cultures in which the parent cell type disappears.

An alteration in the opposite direction has been reported by WESTWOOD ET AL. (1957). Rabbit kidney cells in primary culture are not susceptible to poliovirus. In the course of serial passage, the cells became altered in appearance, multiplied at a more rapid rate, and *became susceptible to poliovirus* (SHEFFIELD AND CHURCHER, 1957). Two American investigators (DREW, 1957; TYTELL, 1957) have confirmed the British work.

Perhaps even more remarkable than the susceptibility to poliovirus acquired by the altered cells is the change in antigenicity of the cells. MELNICK AND HABEL (1958) observed that the altered rabbit cells no longer contained rabbit-species antigens, a finding confirmed by a number of investigators (SABIN, CORIELL, WESTWOOD ET AL., MCLIMANS. While the rabbit kidney passage cells reacted strongly with anti-HeLa and anti-human kidney sera in complement-fixation tests and in cytotoxic antibody tests, they failed to react with anti-rabbit sera. Similar reactions were given by HeLa and monkey kidney antigens. Reciprocally, the anti-HeLa serum yielded similar end points (1:256 to 1:512) when it was titrated against a constant amount of altered rabbit kidney antigen, or HeLa antigen Rabbit kidney antigens failed to react with the anti-HeLa and anti-human kidney sera. Tests for Forssman antigen ruled out the possibility that these results could have been due to its presence in the rabbit and human cells. Whether these cell changes—which are accompanied (1) by a change from resistance to susceptibility to poliovirus (2), by a loss of rabbit antigen, and (3) by the acquisition of a primate antigen—are related in some manner to carcinogenesis is not known. While the altered rabbit cells share antigen with human cancer cells and with human cell lines derived from normal tissues, they failed to react serologically with the S-180 line of mouse cancer cells


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A recent study (KLEINFELD AND MELNICK, 1958) has been carried out on the adjustment and selection of monkey kidney cells leading to *in vitro* growth. The results emphasize the frequency of cellular aberrations that exist even in the primary cultures, the only approved source material for poliomyelitis vaccine used in human beings. The mitotic activity of the primary monkey kidney cultures reached a peak three to five days after seeding, and rapidly declined if the medium was not changed. With replenishment at 48 hour intervals a high mitotic rate was maintained through the eighth day. Cultures subjected to a glycine- and glucose-deficient medium showed a sharp decline in mitotic activity 24 hours later. When such cultures were replenished a burst of mitotic activity occurred 20 to 30 hours later. Mitotic aberrations such as multipolar spindles, anomalous spindles with scattered chromosomes, lagging or precocious movement of chromosomes, chromosome fragments and chromosome bridges were found in all cultures ranging from 16 to 40% of the dividing cells. Non-mitotic aberrations such as giant nuclei, multinucleate cells, nuclear fragmentation, micronuclei, nuclear inclusions, and phagocytosis were found in approximately 3 to 7% of the non-dividing cell population.

Monkey kidney cell cultures have been found to release a proteolytic enzyme, and also an activator of plasminogen, into the culture fluid (BARNETT AND BARON, 1958). Certain lots of commercial poliomyelitis vaccine have been found to contain both the activator and the proteolytic enzyme.

ECHO viruses. Viruses of this group grow preferentially in rhesus and cynomolgus monkey kidney cells. However, they also multiply in human amnion cells (ENDERS, 1957) and Maben carcinoma cells (ORMSBEE AND MELNICK, 1957). Although they usually do poorly in HeLa cultures, they have been adapted to such cells (ARCHETTI ET AL., 1957). Cells of the South American capuchin monkey *(Cebus capucina)* support the growth only of type 10 (RAMOS-ALVAREZ AND SABIN, 1956), and those of the African red grass monkey *(Erythro-*

Fig. 5. Normal monkey kidney epithelial cells, 6 days old. Low power, fixed in Carnoy's fluid and stained with hematoxylin and eosin. (Figures 5–8 are from unpublished data of ORMSBEE, REISSIG, and MELNICK, 1955)

Fig. 6. Monkey kidney cell culture 24 hours after inoculation with ECHO-1 virus. Rounded infected cells are scattered throughout the field.

Fig. 7. A more advanced stage of the infection (66 hours after inoculation) than that in Figure 6. Infected cells are seen as foci of rounded, densely-staining cells. Infected cells are also scattered singly and in small groups throughout the cell sheet.

Fig. 8. A late stage (90 hours after inoculation) of the ECHO-1 infection seen in the preceding photographs. Many infected cells have left the glass. Most of the remaining cells are rounded due to virus infection, although an appreciable number of normal-appearing cells are still present.

cebus patas) only of types 7, 8, and 12 (HSIUNG AND MELNICK, 1957b). Cells of the African green and tantalus monkeys *(Cercopithecus aethiops sabaeus* and *C. aethiops tantalus* are as susceptible, if not more so, for the types tested (DROUHET, 1955, HSIUNG AND MELNICK, 1957b).

The pattern of infection in tissue cultures has recently been summarized (ORMSBEE AND MELNICK, 1957). In most cases the appearance of initial focal lesions is followed by the appearance of widely distributed infected cells around which secondary lesions subsequently develop. (See Figures 5 through 8). With some types the number of lesions remain small. The infection appears to spread largely by cell-to-cell contact, and only occasionally to produce new and separate foci of infection. In such instances the majority of the cells in cultures receiving even undiluted stock virus never seem to be infected by the time the end point in the titration has been reached. The cytologic picture seen in ECHO virus infections of cells in culture is, by and large, indistinguishable from that seen in similar infections with poliovirus, or with those Coxsackie types which grow in monkey kidney cells (BARSKI ET AL., 1955; DUNNEBACKE, 1956; REISSIG ET AL., 1956).

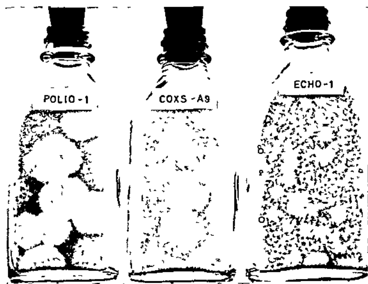


Fig. 9 Rhesus bottle cultures of poliovirus type 1 (Mahoney), Coxsackie virus A-9 (Grigg), and ECHO virus type 1 (Farouk) showing characteristic plaque morphology

Plaques of ECHO viruses on rhesus kidney monolayers usually appear a few days later than those of poliovirus and Coxsackie virus (HSIUNG AND MELNICK, 1957a) Plaques of ECHO viruses are irregular in shape and their boundaries are diffuse, except for types 7, 12, and certain strains of type 8, which produce large, clear plaques

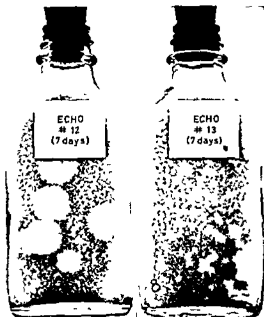


Fig 10 Plaques in monkey kidney monolayer cultures under agar produced by ECHO-12 (Group B), and ECHO-13 (Group A), 7 days after seeding Plaques of ECHO-12 resemble those of wild strains of poliovirus

with sharp boundaries, resembling those of poliovirus It is noteworthy that it is the patas-positive strains which produce the poliovirus-like plaques on rhesus cultures, and ECHO viruses with these two characters have been placed in group B, as against the ECHO viruses in the major group, A (See Figures 9 and 10)

Certain ECHO and Coxsackie viruses agglutinate human Group 0 erythrocytes GOLDFIELD ET AL. (1957) found that the hemagglutinins are associated with the infectious virus particle, for they are sedimented together in the ultracentrifuge, and also they are ad-

sorbed together by erythrocytes during the process of agglutination. The virus and agglutinin may be eluted and in the process, the red cells become exhausted. ECHO viruses may be divided into two groups: one in which the hemagglutinin titer is unaffected by carrying out the reaction at 37°C as against 25°; and a second in which the titer at 37° is greatly reduced. It is of interest that the viruses *with the highest hemagglutinin* titer (over *1:1000*) retain their hemagglutinating power at 37° and that these viruses belong to the group B viruses proposed by HSIUNG AND MELNICK (1957b).

Comparative susceptibility of cells of two monkey species, rhesus and patas, to poliomyelitis, Coxsackie, and ECHO viruses. Table V shows a comparison of rhesus and patas cell susceptibility to poliovirus type 1, Coxsackie virus types A-9 and B-1, and ECHO virus types 1 and 7. This indicates that the same dose of polio-1 or of Coxsackie B-1 *yields twice as many plaques on patas cells as on rhesus; ECHO-7* yields equal numbers of plaques on cells of both species, whereas Coxsackie A-9 and ECHO-1 fails to produce typical plaques in

Table V Susceptibility of Rhesus and Patas Cells to Representative Enteroviruses +

Virus	Virus Dilution	Rhesus		Patas	
		Plaques per bottle	Log titer	*Plaques* per bottle	Log titer
		(Av no)	(PFU/ml)*	(Av no)	(PFU/ml)
Polio-1	$10^{-5.7}$	8.9	7.6	23.5	8.1
Coxsackie A-9	$10^{-6.7}$	18.5	8.9	0	
	$10^{-1.0}$			0**	<1.0
Coxsackie B-1	$10^{4.3}$	17	6.5	33.5	6.8
ECHO-1	$10^{-6.0}$	24.2	8.4	0	
	$10^{-1.0}$			0**	<1.0
ECHO-7	$10^{-5.0}$	25.3	7.4	23.0	7.4

* Plaque-forming units
** With cultures from certain lots of patas monkeys (approximately 20%), about 100 tiny, delayed, slow-growing plaques have appeared with the large dose of virus. It has not been possible to pass viable virus into patas cells from such plaques, although virus could be recovered by transfer of the plaque extract to rhesus cell cultures.
+ From HSIUNG AND MELNICK, *1958a.*

patas cultures. In exploring the reasons for the differences in susceptibility of kidney cells from rhesus and patas to the three groups of enteroviruses, the adsorption of these viruses to cells of both monkey species was investigated (see Table VI) (HSIUNG AND MELNICK, 1958a).

Poliovirus, which yields a higher plaque count on patas cells than on rhesus, was adsorbed by patas to about the same degree as were the two viruses Coxsackie A-9 and ECHO-1, which failed to produce plaques on patas cultures. Obviously the difference between the high susceptibility of patas to poliovirus and its extremely low susceptibility to the Coxsackie A-9 and ECHO-1 must be due to some factor other than adsorption of virus particles to the cells

In patas cells, neither ECHO-1 nor Coxsackie A-9 virus produced plaques under agar, nor cytopathic changes in fluid medium when the virus inoculum was used in low concentration. The virus disappeared within an hour after the inoculation of as much as 500 rhesus plaque-forming units (PFU) of Coxsackie A-9 virus and could not be detected thereafter in either the fluid phase or the cells. That this disappearance was not solely due to thermal inactivation at 37°C is shown by the fact that virus inoculated into control tubes without cells did not become completely inactivated until 24 to 48 hours after inoculation But when the inocula were large, both viruses grew in patas cultures, to a limited degree. It is interesting to note that although the virus yield in patas cultures was less than in rhesus, the shapes of the growth curves were similar in both monkey species.

Although plaques failed to form in cultures of most patas monkeys even with an inoculum of 10 million virus particles (as measured by plaque-forming units on rhesus monolayer cultures), occasionally tiny, delayed, atypical plaques did appear in cultures from some patas monkeys. This phenomenon resembles the physiological restriction of multiplication of attenuated strains of poliovirus—appearance of tiny, delayed plaques under agar overlay containing low concentration of bicarbonate (VOGT ET AL., 1957; HSIUNG AND MELNICK, 1958b) Single Coxsackie A-9 and ECHO-1 plaques of the tiny, delayed type on patas cells were picked but failed to produce virus in patas cultures; however, virus could be recovered in rhesus cultures. Since the poliomyelitis, Coxsackie A-9, and ECHO-1 viruses used in these experiments had been plaque-purified, mixed viral population could not be responsible for the observations reported.

Coxsackie viruses Of the nineteen members of group A, only A-9 grows in kidney cultures of macaque monkeys (see Figure 9),

Table VI Rate of Adsorption of Poliovirus, Coxsackie, and ECHO Viruses on Rhesus and Patas Cells°

Virus	Adsorption Time	Average Number of Plaques on Rhesus Bottles			Av No Plaques on Patas Bottles	Per cent Adsorption on Rhesus*	Per cent Adsorption on Patas**
		Virus adsorbed on rhesus	Free virus from rhesus	Free virus from patas			
	(min)						
Polio-1	30	8 3	8 3	8 7	13 0	61	59
	60	10 5	6 0	5 3	18 1	71	75
	120	20 3	4 8	2 5	30 3	77	88
	120	21 0***	Not removed				
Coxsackie A-9	15	26 8	12 0	13 2	0	63	67
	30	38 3	8 6	7 3	0	79	82
	60	32 2	8 8	7 3	0	78	82
	120	40 0***	Not removed				
ECHO-1	30	34 0	7 0	8 6	0	83	79
	60	38 8	2 2	4 6	0	94	89
	120	40 2	5 0	4 2	0	88	90
	120	40 0***	Not removed				

$$\text{* Per cent adsorption on rhesus} = \frac{\text{Total virus*** minus free virus from rhesus}}{\text{Total virus}}$$

$$\text{** Per cent adsorption on patas} = \frac{\text{Total virus*** minus free virus from patas}}{\text{Total virus}}$$

*** Total virus = Rhesus plaque-forming units at 120 minutes in cultures where free virus was not removed

° From HSIUNG AND MELNICK, 1958a

and even this type fails to grow in cells of the African red grass monkey (HSIUNG AND MELNICK, 1957b). HeLa cells support the growth of group A types 11, 13, 15, and 18 (SICKLES ET AL., 1955). The Coxsackie B viruses grow more readily in cultures; all five types grow in monkey and in HeLa cells, and recently pig kidney cells have been found susceptible (GUERIN AND GUERIN, 1957). Earlier, STULBERG ET AL. (1954) had found that Coxsackie B-1, in contrast to Coxsackie A-1, was cytopathogenic for mouse cells in culture.

Coxsackie B viruses multiply in HeLa cells even when these are transplanted back to the peritoneal cavity of rats. On consecutive passage through the solid HeLa carcinomata which develop in the rats, the viruses develop an increased capacity for destroying these solid tumors (SUSKIND ET AL., 1957). HUEBNER has raised the interesting possibility of removing cancer tissue from human beings, adapting it to grow in rats, and then adapting the Coxsackie virus to it. Once the virus is adapted to the tumor and is able to destroy it completely in rats, it might then be tried out in the patient. If the virus is tried in patients before adaptation, the process might not be nearly as effective in its oncolytic activity in man.

Mixed infections between the enteroviruses. Almost all laboratories engaged in isolating enteroviruses from human beings have encountered specimens containing more than one type of enterovirus. Because these viruses have common properties, BENYESH ET AL. (1957) attempted to determine whether recombination might occur between viruses of each group. The original work—suggesting phenotypic mixing between poliovirus type 1 and ECHO-1 virus—has been reported in summary fashion (MELNICK, 1957b), and the work has since been continued to include studies by the single cell technic of double infections of cells with ECHO-7 and Coxsackie A-9 viruses (ITOH ET AL., 1958).

Monkey kidney monolayer cultures were infected with both viruses. During the eclipse phase the cells were dispersed with versene and single cells isolated in microdrops under oil. Incubation was then allowed to proceed to virus production. The viruses from the individual cells, and their progeny, were characterized serologically and by their pathogenicity to newborn mice. True genetic recombinants could not be detected, but phenotypic mixing did occur. Some cells produced marked doubly antigenic virus, while others gave intermediate results or yielded only the parent ECHO or Coxsackie virus. The original microdrop fluids were plated under agar without anti-

serum, and the resulting plaque extracts were again characterized serologically. Plaques containing both parental types were obtained upon passage of the virus with double antigenicity. The doubly antigenic particles in the single cell harvests behaved as phenotypic mixtures, for they segregated into particles of parental types during the multiplication cycles attendant to plaque formation. Inheritable changes in antigenicity were not found among one thousand specimens of progeny obtained by plaque passage. When tested for mouse pathogenicity, the results were consistent with the antigenic findings: viruses which typed as Coxsackie A-9 were paralytogenic for mice, while those which typed as ECHO-7 were not.

In work of this kind, it is of the utmost importance to have readily distinguishable genetic markers for each virus. The advantage of using plaque morphology as well as antigenic markers for detecting



Fig. 11. Use of rhesus bottle cultures for detecting mixtures of viruses in genetic studies. Plating of progeny of a single plaque (C-100-71) gave plaques characteristic of poliovirus and of ECHO viruses in the bottle on the left, where no antiserum was added. In the bottle second from the left, polio-antiserum was added, neutralizing the poliovirus and leaving only the characteristic small, irregular ECHO plaques. In the next bottle, only ECHO antiserum was added, neutralizing the ECHO virus but leaving the poliovirus unneutralized. In the bottle on the right, antisera to both viruses were added, and both viruses were neutralized.

mixtures in the progeny of single plaques is illustrated in Figure 11 (BENYESH ET AL., 1957). Plaques of two types were present in the progeny of a single plaque. The large plaques were extinguished by polio antiserum and the small plaques by ECHO antiserum.

Size and Morphology of the Enteroviruses

The sizes of these agents have been studied by a variety of methods, including (1) filtration through gradocol membranes of known porosity, (2) determination of sedimentation constants in the ultracentrifuge, (3) direct examination of purified preparations in the electron microscope, and (4) determination of rate of inactivation by ionizing radiation. Although only a small number of the enteroviruses have been studied, and only poliovirus by all of these methods, the available data indicate that the enteroviruses, except for ECHO-10, have a size about 25 to 29 mμ in diameter. The viruses which have been studied in addition to the polioviruses include Coxsackie

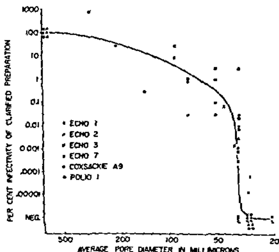


Fig 12 Filtration of ECHO, Coxsackie, and polioviruses through gradocol membranes The limiting A P D (average pore diameter) is 38 mμ The diameter is calculated as 0.64 of the limiting A P.D For the enteroviruses, this calculation yields 24 mμ

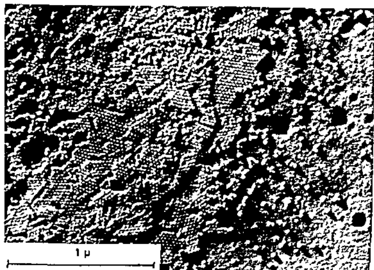


Fig 13 Electron micrograph of Coxsackie A-10 virus Most of the virus particles are packed in crystalline arrays in which each particle measures 28 mμ in diameter Free spherical particles of virus are seen on the right (BREESE AND BRIEFS, 1953)

viruses A-1, 2, 4, 9, and 10, B-1, 2, and 3, and ECHO viruses 1, 2, 3, 7, 8, 9, 10, and 11 (HIMMELWEIT ET AL, 1950; MELNICK ET AL, *1951*; BREESE AND BRIEFS, *1953*; SABIN *ET AL*., *1954*; SCHWERDT, 1957, BENYESH ET AL., 1958; RAMOS-ALVAREZ, 1958).

The results of one series of ultrafiltration experiments on a number of enteroviruses (ECHO, Coxsackie, and polioviruses) are plotted in Figure 12 All of the viruses behaved in a similar fashion and a single line has been drawn through the points. If a factor of 0.64 is used to convert limiting pore diameter (that which just allows a virus to pass) to estimated particle size (BLACK, 1958), the diameters of these enteroviruses are all about 24 mμ Infective particles passed through pores having diameters larger than 38 mμ, but they were held back completely or to some degree by 38 mμ APD membranes. Except for ECHO-7, which had unusually high initial titers, all filtrates from 30 mμ APD membranes were negative.

Ultracentrifugation in the partition cell of an analytical rotor, or through sucrose gradients in tubes in an angle rotor, yielded sedimentation constants of about 150 to 175 S and diameters of about 25 to 30 mμ for poliovirus and for Coxsackie A-1, 2, 4, and 10, and B-1, 2, and 3 strains (MELNICK ET AL., 1951; BREESE AND BRIEFS,

1953). Direct examination of purified preparations of Coxsackie A-2, 4, and 10, B-3, and of the polioviruses in the analytical ultracentrifuge and in the electron microscope yielded values of 28 mμ (see Figure 13) (BREESE AND BRIEFS, 1953; MATTERN AND DUBUY, 1956; TAYLOR AND MCCORMICK, 1956; SCHWERDT, 1957)

Polioviruses have recently been obtained in crystalline form (SCHWERDT, 1957), and this also has been achieved with one of the Coxsackie viruses, A-10 (MATTERN AND DUBUY, 1957). The viruses are spherical in shape, and form crystals on storage in the cold (see Figure 14). As for the Coxsackie A-10 virus, the crystalline material has a titer of 10^{13} LD_{50} per cubic centimeter. As this volume contains about 4 6 × 10^{16} particles of 28 mμ diameter, the ratio of number of particles to number of infective units in the crystal was about 4600 to 1 Both the purified poliomyelitis and the purified Coxsackie viruses contained nucleic acid of the ribose type. Evidence is beginning to accumulate that, as with tobacco mosaic virus, the viral infective unit is contained in the nucleic acid part of the virus particle. A full discussion of this feature will be found in the chapter by COLTER in this volume.



Fig 14 Crystals of Coxsackie A-10 virus under dark-field illumination (MATTERN and DUBUY, 1956)

The methods of ultrafiltration, ultracentrifugation, and electron microscopy are now standard practice in virus laboratories, but that of ionizing radiation (POLLARD ET AL., 1955) has not been widely used. An advantage of ionizing radiation methods over electron microscopy is the direct relationship between the biological activity of the unit and the experimental observations. There are advantages over ultrafiltration and ultracentrifugation in precision, and also in the fact that one can determine by ionizing radiation how large a portion of the virus particle is concerned with various functions. The results obtained by irradiation methods are not entirely comparable to those obtained by electron microscopy, ultrafiltration, or ultracentrifugation because the size determined by ionizing radiations gives the size of a unit, in which damage to any part will destroy the activity under consideration. This radiation-sensitive unit does not necessarily correspond to the whole of the particle measured by other methods.

If there are radiation sensitive properties other than that of infectiousness, the rate of loss of each property will be proportional to the size of the unit which governs the property in question In this way

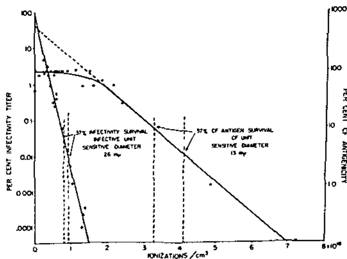


Fig 15 Effect of high energy electron irradiation on two properties of ECHO-1 virus: infectivity and complement-fixing activity. From the amount of energy necessary to bring each activity to the 37% survival point, the sensitive diameters were calculated to yield the results shown on the chart

the size of the complement-fixing antigens within the virus particle, as well as that of the infectious unit, has been determined for several enteroviruses (BENYESH ET AL., 1958).

Figure 15 illustrates the inactivation by high energy electrons, at 4°C, of two different properties of ECHO-1 virus: infectivity, and complement-fixing antigenicity. Their inactivation is plotted on the same dosage scale, but two different activity scales are used. The diameter of 26 mμ for the infective unit was determined by calculating the amount of energy necessary to bring the infective component to the 37% survival point.

The plot of virus infectivity on a logarithmic scale versus dose of irradiation gave a straight line, indicating one hit per inactivation. In contrast to the infectivity plot for this virus and also to the complement-fixing activity for poliovirus, the plot of residual complement-fixing activity for ECHO-1 had a definite shoulder. The descending portion of the curve indicates a diameter of 13 mμ for the sensitive portion of the complement-fixation antigen. Extrapolation of the descending portion of the curve to the ordinate gives an intercept at about 600%

The inactivation data of different units of a virus particle may give important leads as to its structure. For example, the size of the complement-fixing antigenic units of all the enteroviruses studied fell into a smaller size range (7 to 13 mμ) This is about the size of a moderately large protein. Thus the CF antigen appears to be either a moderately large structure or else a labile group attached to a unit along which a charge can be conducted It is not possible, on the basis of the present experiments, to say whether these units are independent of the infective unit or a portion thereof. The initial slow rate of inactivation of the complement-fixing antigen of ECHO-1 has led to the following interpretations:

1. During the first portion of the process new antigenic units may be formed—possibly by separating or splitting original ones. This process would be at first balanced by and later supplanted by inactivation.

2. There may be several antigenic units on the virus particle spaced so closely that only about 1 in 6 can react with complement Thus until all 6 in a given area (or perhaps this is the total per particle) are inactivated, the complement binding power is unaffected.

3. The antigenic unit may be more resistant to inactivation than the infective unit, and the complement binding power lost only after the unit has been hit about 6 times.

Clinical Features

Infections produced by the enteroviruses vary from the very serious diseases, such as paralytic poliomyelitis, to the very mild, subclinical infections produced by many of the Coxsackie and ECHO viruses. The views of the Committee on the Enteroviruses (1957) in this regard have recently been set forth as follows:

"For establishment of etiologic association the virus must have a much higher prevalence among patients with the disease than in healthy individuals of the same age and socio-economic status living in the same area at the same time as the patients. Antibodies against the virus must develop during the course of the illness. Virologic or serologic evidence must be negative for concurrent infection with other agents that already are known to cause the same clinical syndrome. Etiologic probability is increased if the virus is isolated ni significant concentration from body fluids or tissues manifesting the lesion, as from the cerebrospinal fluid in cases of aseptic meningitis, or the heart muscle in cases of myocarditis."

Of the three types of poliovirus, type 1 continues to be the agent most often associated with clinical disease. With the continued improvement and simplification of tissue culture methods, it has been possible to put together a simple portable tissue culture "laboratory" which is adequate for the study of epidemics of poliomyelitis in any part of the world accessible by air travel (MELNICK, 1958).*

As regards Coxsackie viruses, all 5 types belonging to group B have been found to be associated with clinical illness. Of special importance are the recent associations of the group B viruses with neonatal myocarditis, as first shown by the South African workers, and reviewed elsewhere in this volume by Dr. GEAR. With group A, only A-9 has been regularly associated with aseptic meningitis, even though A-7 has been at times.

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* For example, it was possible to carry tissue cultures and diagnostic reagents into the first epidemic occurring in British Guiana, in 1957. In the course of one week's stay in the area, it was found (1) that type 1 poliovirus was the agent responsible for the outbreak, although type 2 was also active, (2) that other enteroviruses were infecting some of the patients diagnosed as having poliomyelitis, and (3) that type 1 poliovirus was prevalent but to a lesser degree among control children of the same age as those afflicted with clinical poliomyelitis. Serological tests in the field were limited to the performance of complement fixation tests with poliovirus antigens

Evidence of the causal relationship of Coxsackie viruses B-1 to B-5 and A-9 to aseptic meningitis has recently been strengthened by the isolation of all 6 Coxsackie viruses from the spinal fluids of aseptic meningitis cases (data reviewed by DALLDORF, MELNICK, AND CURNEN, 1958). The question has been raised as to whether the finding of virus in the spinal fluid always implicates the virus as the agent of the disease. In severe diseases, the blood-brain barrier might be lowered to such an extent that the virus might be present in the spinal fluid merely as an incidental finding. Thus, the finding of Coxsackie virus in the spinal fluid of two patients with brain tumors is of particular interest (BENYESH AND GOLDBLUM, 1955). The question has been raised as to whether the Coxsackie virus, which is known to have an affinity for newborn tissue, found young cells in the brain tumor in which it was able to multiply.

Meningitis induced by Coxsackie virus may often be complicated by the fact that pleurodynia occurs in the same patients. Thus, in some epidemics the patients may exhibit pleurodynia alone, meningitis alone, or both manifestations, all caused by the same virus

Recent work from the Soviet Union has indicated that type A-7 may also be associated with paralysis in children. The Soviet workers isolated a virus which produced paralysis and lesions in monkeys typical of poliomyelitis, and also showed that this virus could be passed in rodents (CHUMAKOV ET AL., 1956) The Russian strains of this virus do not appear to be unique, for subsequent to their work, strains isolated in the United States have been tested and found to be pathogenic for both monkeys and suckling mice The A-7 virus has also been associated with aseptic meningitis in Sweden and in the United States (JOHNSSON AND LUNDMARK, 1957).

As regards the group A viruses, types A-7 and A-9 have been associated with aseptic meningitis, as mentioned above. However, the most common disease caused by the group A viruses is herpangina, a syndrome produced by types 2, 4, 5, 6, 8, and 10 (HUEBNER ET AL., 1952).

Many members of the ECHO group have been found to cause illness. ECHO-4, first isolated from patients with aseptic meningitis (MELNICK ET AL., 1953), has been found to be responsible for large outbreaks of this disease (CHIN ET AL., 1957), as well as for sporadic cases in other areas (ORMSBEE AND BELL, 1957). A rash was observed in some of the patients in a Swedish epidemic of ECHO-4 aseptic meningitis (JOHNSSON, 1957). ECHO-6 has also been found to cause

epidemic aseptic meningitis (KARZON ET AL., 1956; DAVIS AND MELNICK, 1956; VON ZEIPEL AND SVEDMYR, 1957; JOHNSSON ET AL., 1957; HABEL ET AL., 1957; KIBRICK ET AL., 1957). In some of the cases, muscle weakness and mild to moderate paralysis were observed; with the passage of time, recovery was usually complete.

The recent widespread outbreaks of ECHO-9 infections involving thousands of patients gave investigators an opportunity to record its clinical behavior (NIHOUL AND QUERSIN-THIRY, 1957; BOISSARD ET AL., 1957; JOHNSSON, 1957; MCLEAN AND MELNICK, 1957; BAUMANN ET AL., 1957; GODTFREDSEN AND VON MAGNUS, 1957; SABIN ET AL., 1958; and others). ECHO-9 may produce the following clinical pictures: aseptic meningitis, undifferentiated febrile illness which may be accompanied by an exanthem and enanthem, or—rarely—a more extensive involvement of the central nervous system. The incidence of rash decreases with age. Thus SABIN ET AL. (1958) found that rash occurred in 70% of patients under 4 years of age, in 44% in those 5–15 years of age, and in only 7% of patients over 15 years of age. Conjunctivitis may also be present. Virus may be detected in the spinal fluid even in the absence of pleocytosis. ECHO-9 virus may be responsible for illnesses more severe than that usually associated with aseptic meningitis. Muscle weakness and spasm may persist for weeks. The virus has been recovered in high titer from the medulla of an infant dying within a day after becoming ill with fever and coma (VERLINDE, 1957).

ECHO-10 has been associated with steatorrheic enteritis in humans and with a common-cold coryzal syndrome in chimpanzees (SABIN, 1957), but the precise clinical pattern remains to be determined.

ECHO-16, like some of the other ECHO viruses mentioned, may also produce aseptic meningitis (KIBRICK ET AL., 1957). NEVA (1957) has shown that the etiological agent of Boston exanthem (NEVA ET AL., 1954; NEVA AND ZUFFANTE, 1957) is antigenically related to ECHO-16 virus.

ECHO-18 has been reported to cause infant diarrhea (RAMOS-ALVAREZ AND SABIN, 1958; EICHENWALD ET AL., 1958). In older persons, it has not been associated with illness. Evidence has been obtained (RAMOS-ALVAREZ AND SABIN, 1958) to suggest that in young children different ECHO viruses (especially types 6, 7, 8, 12, 14) may cause diarrheal disease, and ECHO-20 a febrile disease involving both the respiratory and enteric tracts (ROSEN ET AL., 1958). ECHO types 2, 3, 5, and 14 have also been associated with aseptic

meningitis cases, with types 5 and 14 having been isolated from the cerebrospinal fluid (MELNICK, 1955, 1957b; ORMSBEE AND BELL, 1957).

For many of the enteroviruses, no disease entities have yet been described. However, even the virulent enteroviruses are characterized by the fact that they produce large numbers of inapparent infections. The variety of disease entities produced by the enteroviruses is shown in Table VII

The enteroviruses have been studied in the highest laboratory primate available, the chimpanzee. The pattern of infection of these anthropoids is the same with the polioviruses (HOWE ET AL., 1950; MELNICK AND HORSTMANN, 1947); the Coxsackie viruses (MELNICK AND KAPLAN, 1953); and the ECHO viruses (ITOH AND MELNICK, 1957) Illness was a rare event in these orally infected animals, but

Table VII Diseases Associated with Enteroviruses

Enteroviruses	Associated Diseases
Polioviruses	Paralysis (complete to slight muscle weakness) Aseptic meningitis Undifferentiated febrile illness particularly during the summer
Coxsackie viruses Group A	Herpangina (Types 2, 4, 5, 6, 8, 10) Undifferentiated febrile illness particularly during the summer Aseptic meningitis (Types A-7, A-9) Febrile illness with rash (Type A-9)
Coxsackie viruses Group B	Aseptic meningitis Pleurodynia (Bornholm disease) Undifferentiated febrile illness with pharyngitis Myocarditis or encephalomyocarditis during neonatal period and early childhood Mild paralysis (?) or encephalitis
ECHO viruses	Aseptic meningitis (Types 2, 3, 4, 5, 9, 14, 16, 21) Febrile illness with rash (Types 4, 9, 16, and probably others) Boston exanthem (Type 16) Undifferentiated febrile illness particularly during the summer Mild paralysis (?) (Types 6 and 9) or encephalitis (Type 9) Summer diarrhea of infants and children (Type 18 and others)

epidemic aseptic meningitis (KARZON ET AL., 1956; DAVIS AND MELNICK, 1956; VON ZEIPEL AND SVEDMYR, 1957; JOHNSSON ET AL., 1957; HABEL ET AL., 1957; KIBRICK ET AL., 1957). In some of the cases, muscle weakness and mild to moderate paralysis were observed; with the passage of time, recovery was usually complete.

The recent widespread outbreaks of ECHO-9 infections involving thousands of patients gave investigators an opportunity to record its clinical behavior (NIHOUL AND QUERSIN-THIRY, 1957; BOISSARD ET AL., 1957; JOHNSSON, 1957; MCLEAN AND MELNICK, 1957; BAUMANN ET AL., 1957; GODTFREDSEN AND VON MAGNUS, 1957; SABIN ET AL., 1958, and others). ECHO-9 may produce the following clinical pictures: aseptic meningitis, undifferentiated febrile illness which may be accompanied by an exanthem and enanthem, or—rarely—a more extensive involvement of the central nervous system. The incidence of rash decreases with age. Thus SABIN ET AL. (1958) found that rash occurred in 70% of patients under 4 years of age, in 44% in those 5–15 years of age, and in only 7% of patients over 15 years of age. Conjunctivitis may also be present. Virus may be detected in the spinal fluid even in the absence of pleocytosis. ECHO-9 virus may be responsible for illnesses more severe than that usually associated with aseptic meningitis. Muscle weakness and spasm may persist for weeks. The virus has been recovered in high titer from the medulla of an infant dying within a day after becoming ill with fever and coma (VERLINDE, 1957).

ECHO-10 has been associated with steatorrheic enteritis in humans and with a common-cold coryzal syndrome in chimpanzees (SABIN, 1957), but the precise clinical pattern remains to be determined.

ECHO-16, like some of the other ECHO viruses mentioned, may also produce aseptic meningitis (KIBRICK ET AL., 1957). NEVA (1957) has shown that the etiological agent of Boston exanthem (NEVA ET AL., 1954; NEVA AND ZUFFANTE, 1957) is antigenically related to ECHO-16 virus.

ECHO-18 has been reported to cause infant diarrhea (RAMOS-ALVAREZ AND SABIN, 1958, EICHENWALD ET AL., 1958). In older persons, it has not been associated with illness. Evidence has been obtained (RAMOS-ALVAREZ AND SABIN, 1958) to suggest that in young children different ECHO viruses (especially types 6, 7, 8, 12, 14) may cause diarrheal disease, and ECHO-20 a febrile disease involving both the respiratory and enteric tracts (ROSEN ET AL., 1958). ECHO types 2, 3, 5, and 14 have also been associated with aseptic

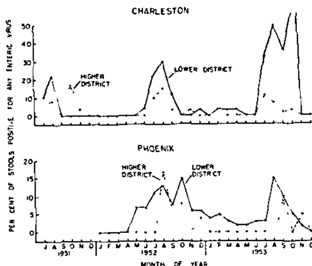


Fig 16 Per cent of stool specimens yielding enteric viruses, each month over a 3-year period (1951 through 1953) Specimens were obtained from healthy children in contrasting socio-economic districts of Charleston, W Virginia, and Phoenix, Arizona, during a nonepidemic period

almost all of the virus isolations were made in the summer and fall, except from the lower socio-economic district of subtropical Phoenix, where viruses were recovered more evenly throughout the year.

The frequency of virus excretion in the lower socio-economic district of each city was 3 to 6 times as great as in the middle to upper middle class districts with good environmental sanitation. If the combined figures are examined (Table IX), of 1540 specimens tested from the lower districts, 176 or 11 4 % were positive, whereas from the upper districts only 2.6 % (37 of 1427 specimes tested) yielded virus. Among the 213 viruses isolated in monkey kidney cultures, 52 % were ECHO viruses, 24 % were polioviruses, and 24 % were Coxsackie viruses.

HAMMON and his colleagues (1957) have recently carried out a study of infection rates of two enteroviruses in a selected area in the Philippine Islands Age specific rates were calculated from the number of persons who developed neutralizing antibody during a four month period of observation when enteroviruses were circulating silently. The infection rates for children were 90 % for ECHO-1 and

infection was readily demonstrated by the presence and persistence of virus in the throat and in the feces, and by the type-specific antibody response. It is noteworthy that with enteroviruses, virus may sometimes be found in the throat for as long as, or even longer than in the intestine (Coxsackie B-2, ECHO types 4 and 6).

Epidemiology

The enteroviruses have a global distribution. Recent investigations with the Coxsackie and ECHO viruses show that their epidemiologic patterns are similar to those of the polioviruses. For example, the carrier rate for the ECHO viruses decreases as age increases. Thus, as shown in Table VIII, in Cincinnati 5.2 % of children aged 1–4 years excreted virus, but only 0.2 % of those 10–14 years of age (RAMOS-ALVAREZ AND SABIN, 1956). The difference in rates between the very young in Cincinnati and in Mexico City is similar to that which has been observed in children living under contrasting socio-economic environments within single cities. In a longitudinal study (HONIG ET AL., 1956; ISACSON ET AL., 1957) of the endemic occurrence of enteroviruses among several hundred normal households, approximately equal numbers of stools were collected each month from children under the age of 5 years. As shown in Figure 16,

Table VIII Isolation of Enteroviruses from Rectal Swabs of Healthy Children in Mexico City and Cincinnati*

City	Age Group	Number of Children Tested	Virus Isolated	
			Polioviruses	Coxsackie and ECHO Viruses
	(yrs)		%	%
Mexico City	1–4	1491	3 4	15 6
Cincinnati	1–4	154	0 6	5.2
	5–9	537	0 3	2 6
	10–14	683	0 2	0 2
	15–17	154	0 0	0 0

* Data from RAMOS-ALVAREZ AND SABIN (1956)

summer months was not constant, but varied in the same community from one year to the next, ranging anywhere from 10 to 100 per cent. The changing annual pattern was not only related to the frequency of virus occurrence but also to the type. For example (MELNICK ET AL., 1954) in one area, Muskegon, Michigan, of 34 Coxsackie strains isolated in sewage during the summer and fall, 26 belonged to a single type, A-1. On the other hand, in Topeka, Kansas, A-5 predominated in the first part of the warm season, to be replaced by A-6 in the fall of the same year. In Albany (KELLY, 1957), in one year A-5 and A-6 were the predominant types, a year later, they had been replaced by types A-4, A-8, and B-2. Thus this method of enterovirus surveillance allows one to determine when a new virus becomes disseminated in a community. The available data have already shown that the persistence of an enterovirus for several months during the summer and fall season may actually be a series of waves of different types spreading through the city

A recent two-year study of aseptic meningitis cases in Connecticut (DAVIS AND MELNICK, 1958) indicated the extent to which different

Table X Distribution of Enterovirus Isolations from Cases of Aseptic Meningitis in Connecticut

Viruses Isolated	Number of Isolations		
	From 137 cases studied in 1955	From 82 cases studied in 1956	From total of 221 cases
Poliovirus Total	28	6	34
Type 1	27	4	31
Type 3	1	2	3
Coxsackie Total	15	13	28
A-9	1	6	7
B-2	5	1	6
B-3	2	4	6
B-4	7	2	9
ECHO Total	25	11	36
Type 5	1	0	1
" 6	22	9	31
" 14	2	2	4
Total	68	30	98

Table IX. Per Cent Distribution of Enteric Viruses Isolated from Healthy Children in Populations of Contrasting Socio-Economic Level During a Nonepidemic Period (1951–53)

Population Group	Number of *Specimens* Tested	Per Cent Yielding Viruses			
		Polio-viruses	Coxsackie viruses	ECHO viruses	All enteric viruses
Charleston, W Va					
Lower	597	2.3	2.3	3.7	8.4
Upper	1028	0.5	1.5	0.8	2.7
Phoenix, Ariz					
Lower	943	3.0	2.0	8.3	13.3
Upper	399	1.0	1.0	0.3	2.3
TOTAL:					
Lower	1540	2.8	2.1	6.6	11.4
Upper	1427	0.6	1.3	0.6	2.6

70 % *for poliovirus, for adults these were 40 and 36 %*, respectively. The results were similar to those observed earlier during an epidemic period in North Carolina (MELNICK AND LEDINKO, 1953), when matched sera from healthy children were obtained at the beginning and at the end of the epidemic, and analyzed for a variety of antibodies. High infection rates were found for polioviruses type 1 (45 % and 7 % for lower and upper socio-economic groups, respectively), type 2 (26 % and 14 %), and a local Coxsackie virus, A-4 (33 % and 46 %). That the results were specific is shown by the fact that there was almost no evidence for infection with other agents (type 3 poliovirus, mumps virus, influenza virus, and streptococcus)

Epidemics produced by the enteroviruses occur in the temperate zones during the summer and early fall seasons, although cases may continue into the winter. The seasonal prevalence of enteroviruses is well illustrated in the consecutive tests which have been carried out for enteroviruses in sewage and flies (MELNICK, 1947; RHODES ET AL., 1950; GEAR, 1952; MELNICK AND DOW, 1953; MELNICK ET AL., 1954; KELLY, 1957) In almost every area studied, virus could be found *in some of the specimens which were collected in* the summer and fall seasons. During the cold months, virus was only rarely found, indicating that it was not widely disseminated in the community during this *period. The frequency* of virus recovery even during the

Table XI Neutralizing Antibody Survey for Some *Enteroviruses* Associated with the Aseptic *Meningitis Syndrome* in Connecticut *during* 1955 and 1956*

Virus	In Persons 0–14 Years of Age			In Persons 15 Years of Age and Over		
	Number Tested	Number with Neutralizing Antibodies**	Per cent with Neutralizing Antibodies	Number Tested	Number with Neutralizing Antibodies**	Per cent with Neutralizing Antibodies
Poliovirus***						
Type 1	48	28	58	61	38	61
Type 2	48	17	35	61	34	56
Type 3	48	11	23	61	33	54
Coxsackie Virus						
Type A-9	152	30	20	72	35	49
Type B-1	148	17	11	63	4	6
Type B-2	148	49	33	64	24	38
Type B-3	139	32	23	60	24	40
Type B-4	125	34	27	57	35	61
ECHO Virus						
Type 2	151	28	19	66	24	36
Type 5	151	9	6	61	10	16
Type 6	130	22	17	71	16	23
Type 9	115	14	9	52	10	19

* Neutralizing antibodies from donors whose stool *was negative* or who were excreting a virus other than that related to the antibody under test

** Neutralizing antibodies of a titer of *1:10 or higher versus* 100 TCD_{50} of virus

*** Only non-vaccinated donors were tested

members of the enterovirus group may simultaneously be responsible for the same clinical syndrome. The age, sex, and seasonal distribution, as well as the clinical course of the illness, simulated that of nonparalytic poliomyelitis. As can be seen from the data in Table X, ECHO viruses (types 6 and 14) and Coxsackie viruses (types A-9, B-2, B-3, and B-4) were as frequently isolated from this group of patients as were the polioviruses. Antibody responses were specific for the virus recovered. Similar studies have been carried out by HABEL ET AL. (1957), VON ZEIPEL AND SVEDMYR (1957), KIBRICK ET AL. (1957), and others. In the course of the study in Connecticut, confirmation was obtained of the effect of Salk vaccine in increasing the poliomyelitis antibody prevalence and decreasing the incidence of poliomyelitis in the vaccinated group. In contrast, the vaccine had no effect on the incidence of aseptic meningitis due to Coxsackie or ECHO viruses. In the course of the above study, an enterovirus antibody survey was carried out, which yielded the following results (see Table XI):

Fifty-eight per cent (28 of 48) of the unvaccinated children demonstrated antibodies for poliovirus type 1, while 35 per cent (17 of 48) and 23 per cent (11 of 48) demonstrated antibodies to types 2 and 3, respectively. Even though a higher percentage of the adults was positive, 18 per cent (11 of 61) of the adults tested failed to demonstrate any poliomyelitis antibodies.

The prevalence of Coxsackie A-9 antibodies was essentially the same as for poliomyelitis with 20 per cent of the children and 49 per cent of the adults being positive. For the Coxsackie B viruses, using essentially the same sera, 11 per cent of those under 15 years had B-1 antibodies, 33 per cent B-2, 23 per cent B-3, and 27 per cent B-4, while among the adults, 6 per cent had B-1 antibodies, 38 per cent B-2, 40 per cent B-3, and 61 per cent B-4. Antibodies against 4 ECHO viruses were tested in a similar manner. Among those under 15 years of age, 19 per cent showed ECHO-2 antibodies, 6 per cent ECHO-5, 17 per cent ECHO-6, and 9 per cent ECHO-9, while among the adults the corresponding figures were 36, 16, 23, and 19 per cent respectively.

Studies of the families into which the enteroviruses were introduced demonstrated the ease with which these agents spread, and the high frequency of infection in those without pre-existing antibodies from earlier exposures. This was the case regardless of the viral type under study. Thus the rapid, and usually silent, dissemination of the enteroviruses in households is another property which they share. Hospital-

cimen is tested by the plaque method (see Figure 17). When found together in a patient, it is difficult to say the extent to which each contributed to the overt illness. It is evident that infections by the poliomyelitis, Coxsackie, and ECHO groups have many features in common, and that studies on the epidemiology of any one group require consideration of the other groups

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ized patients may not give a true picture of the extent of illness caused by an enterovirus in a community. Thus a survey during an ECHO-4 outbreak, in which 27 cases of aseptic meningitis were reported in a town of 20000, revealed that 16 % of the population had a compatible illness (LEHAN ET AL., 1957). Similarly, during a period when 149 inhabitants of a city of 740000 were hospitalized with ECHO-9 disease, it was estimated that approximately 5 %, or 45000 persons, had a compatible illness (SABIN ET AL., 1958).

The similarities in the epidemiology of the different enteroviruses may be briefly summarized. Viruses of all three groups induce in man infections associated with specific antibody responses. In the human host, agents of different groups may be carried for some time, with the greatest incidence of carriers among young children. They may cause either recognizable disease or inapparent infection. The enteroviruses are encountered most frequently during the warm seasons of the year. They may be recovered not only from man but also from flies and sewage, and at times viruses of the different groups may be found in the same specimen, as is strikingly revealed when the spe-

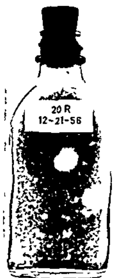


Fig. 17. Culture, inoculated with a stool specimen containing a mixture of polio and ECHO viruses, and overlaid with agar. By picking the large or small plaques for passage, the poliovirus or the ECHO virus, respectively, could be isolated.

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Mice infected with Group B strains develop weakness, tremors, spasms, and paralysis. The pads of fat between the scapulae macroscopically show whitish degeneration; microscopically there is necrosis associated with an acute inflammatory infiltration, which may be followed by calcification of the affected areas. This lesion of the fat pad is characteristic of Coxsackie B virus infections in mice. In addition the brain may show focal lesions with degeneration of neurones, with pyknosis and fragmentation of the nuclei, followed by dissolution or "fall out" of the affected cells. Areas of softening and rarefaction and a perivascular and diffuse infiltration of inflammatory cells may also be seen. The heart muscle often shows foci of necrosis with eosinophilic degeneration of segments of muscle fibres with loss of striation, with pyknosis, and fragmentation of the nuclei and an associated inflammatory cell infiltration.

In weaned mice these viruses usually produce no signs of infection but some strains have been adapted by passage to cause lesions in adult mice. Notable examples are the degeneration and dissolution of the parenchymal cells of the pancreas caused by infection with certain strains of Group B type 3 viruses, and the lesions of the central nervous system caused by certain strains of Group A viruses in both adult mice and monkeys. These viruses may also cause fatal disease in adult mice treated with cortisone.

The pathological findings in mice resulting from infection with the Coxsackie viruses are noted because of their relevance to the infections in human beings, particularly newborn babies.

Coxsackie Group A viruses have been incriminated as the cause of herpangina, of some short pyrexial illnesses, and of some cases of meningo-encephalitis. Extensive epidemics of an illness associated with a rash, lymphadenopathy, and often with aseptic meningitis have been traced to infection with the Coxsackie A-like Echo 9 virus. Whether any of these illnesses are more severe in newborn babies than in older children and adults has not yet been assessed, but it may be expected that newborn babies whose mothers lack immunity will suffer more severely. ARCHETTI AND BORTOLOZZI (1953) have suggested that such infections may sometimes be fatal, and indeed this seems likely. However, the role of Coxsackie A viruses in causing disease in newborn babies has not yet been clearly assessed and certainly merits further study.

Coxsackie Group B viruses have been incriminated as the cause of epidemic myalgia, pleurodynia (Bornholm disease), and as one of

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COXSACKIE VIRUS INFECTIONS OF THE NEWBORN

By JAMES H. S. GEAR

Coxsackie viruses were first isolated in 1947 by DALLDORF AND SICKLES. The isolation was achieved by the inoculation of newborn mice. The pathogenicity of these viruses for newborn mice is one of their characteristics. The relative lack of pathogenicity for adult mice and for other experimental animals accounted for their escape from recognition before this time.

Later studies have revealed that there are 2 groups of Coxsackie virus which have been separated from each other on the basis of the lesions produced in baby mice and named respectively Group A and Group B. Mice infected with Coxsackie Group A virus strains develop flaccid paralysis and on histological examination show eosinophilic hyalin degeneration and acute inflammation of the voluntary striated muscles. Lesions are not seen in the other organs. The diffuse muscle destruction associated with an acute inflammatory infiltration, and an absence of lesions elsewhere is characteristic of Group A Coxsackie virus infections. Nineteen serotypes respectively named A1–19 have been identified.

only one examined a Coxsackie Group B type 3 virus was isolated.

These two outbreaks illustrated the two common serious forms of disease caused by Coxsackie Group B virus in newborn babies. In some the brunt of the infection is borne by the heart and in others by the central nervous system.

A third outbreak to be investigated occurred in a maternity home in Southern Rhodesia (MONTGOMERY ET AL., 1955). Three newborn babies were affected Two recovered after being ill about one week, the third died on the 12th day after birth, and on postmortem examination the heart showed focal myocarditis. A virus identified as Coxsackie Group B type 4 virus was isolated from the faeces and the caecal content of the baby who died and from the faeces of one of the babies who recovered.

In 1955 a Coxsackie Group B type 2 virus was isolated from the heart muscle of a 9 day old baby who died soon after admission to the Transvaal Memorial Hospital for Children, with fever and signs of myocarditis. Sections of the heart showed an extensive focal myocarditis similar to that described in the previous cases. The relationship of Coxsackie Group B virus to this condition was thus proved.

The aetiological role of Coxsackie Group B virus in causing disease, often fatal, in newborn babies was convincingly confirmed in 1955 in Holland by VAN CREVELD AND DE JAGER, by VERLINDE, VAN TONGEREN AND KRET, and by DEKKING. Full clinical, pathological, and virological studies of four cases were carried out. The first of the patients concerned was a 7 day old boy who had been born prematurely one month before term, and who died one day after admission to hospital. Postmortem showed a myocarditis, encephalitis, and hepatitis. The second patient was an 8 day old girl who was admitted to the Clinic in a cyanotic collapsed state and who died the day after admission. Postmortem examination revealed myocarditis and foci of inflammation in the pericardium, pleura, kidney, and brain. The third case was a premature boy admitted to hospital when aged 3 weeks with myocarditis and meningo-encephalitis. This child died 9 days after admission to hospital Postmortem examination confirmed the presence of myocarditis and meningoencephalitis. The fourth case was a boy born at term who suddenly on the 7th day of life collapsed and died one hour later. Postmortem showed the cause of death to be myocarditis. A Coxsackie Group B type 4 virus was isolated from the brain and heart muscle of the first case, and from heart muscle of the other three cases.

the commonest causes of the aseptic meningitis syndrome. These infections in older children and adults are generally presumed to be benign and almost never cause a fatal illness. However, it is now clear that infection with Coxsackie Group B viruses may cause a severe often fatal illness in newborn babies, in which the prominent pathological finding is an acute extensive but focal myocarditis, sometimes associated with focal lesions in the central nervous system, the liver, and other abdominal organs.

Recent Outbreaks of Encephalo-Myocarditis Neonatorum Associated with Coxsackie Group B Virus Infections.

The first evidence incriminating Coxsackie Group B virus as a cause of encephalomyocarditis in newborn babies was given by GEAR AND MEASROCH, who studied an outbreak which occurred in a maternity home in Johannesburg in October and November 1952.

The clinical features of these cases were later described in detail by Javett and his associates, and the pathological features by Pepler and Lurie, and these will be briefly noted. Ten newborn babies became ill whilst in or soon after their discharge from the maternity home in which they were born. They had fever and developed signs and symptoms of acute myocarditis and in some cases signs of meningoencephalitis. Four recovered and apparently have suffered no serious sequelae. Six died after an acute fulminating illness ending in circulatory collapse. Postmortem examination revealed that the cause of death was an acute extensive but focal myocarditis associated in some cases with focal lesions in the brain and occasionally in other organs. Coxsackie Group B type 3 virus was isolated from the faeces of one of the babies who recovered. Some of the baby mice in litters inoculated with suspensions prepared respectively from the heart muscle and brain of two of the fatal cases developed weakness and tremors, and on histological examination showed degeneration and acute inflammation of the fat pad and focal lesions of the brain similar to those of Coxsackie Group B infections.

Shortly after this, in December 1952, another outbreak involving three babies occurred in another maternity home. These babies all showed, in addition to fever and marked tachycardia suggestive of myocarditis, signs of meningo-encephalitis including a pleocytosis of the cerebrospinal fluid. All recovered, and from the faeces of the

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In 1956 KIBRICK AND BENIRSCHKE reported their findings in a study carried out in 1954 of a case in a newborn infant, who died seven days after birth by caesarean section. At birth the condition of this infant appeared good, but several hours later she developed a croupy cough. On the 3rd day her rectal temperature rose to 100.2°F. On the 4th day she was afebrile, but on the 5th day the fever recurred and a lumbar puncture revealed the presence of a pleocytosis and an increased protein content. On the 6th day her condition abruptly grew worse and she collapsed and died on the 7th day.

A complete postmortem examination revealed a diffuse myocarditis and a disseminated encephalomyelitis with an infiltration of cells in the meninges and focal lesions in the cerebellum, pons, and spinal cord. A Coxsackie Group B type 3 virus was isolated from the spinal cord in tissue cultures of human renal cells. They concluded that this infection had been acquired in utero from the mother.

More recently the ENDERS group in Boston have reported briefly the isolation of a Coxsackie Group B type 4 virus from a similar case.

From a review of these studies it is now possible to present a clear composite picture of the epidemiological, clinical, pathological, and virological aspects of this condition of encephalo-myocarditis of newborn babies caused by Coxsackie Group B virus infections.

Epidemiological Features

Season. Most outbreaks and cases have occurred in the warm summer and autumn months when Coxsackie virus as well as poliovirus infections are most prevalent. Two of the Southern African outbreaks and the cases in Holland occurred when Bornholm disease was epidemic. At such times opportunities for the contamination of maternity homes will occasionally occur.

Source of infection. Most commonly the infection appears to have been introduced by an expectant mother, occasionally by a member of the nursing staff. The babies acquire the infection either directly from their mothers, or from a member of the nursing staff or from other babies in the nursery.

In the outbreak reported from Southern Rhodesia and in the cases reported from Holland, the mothers or some other member of the family had suffered from a short pyrexial illness shortly before

or shortly after their babies' birth. Retrospectively this illness was considered to be due to Coxsackie Group B virus infection.

In the case reported from Boston, the mother concerned was ill with an upper respiratory infection with coryza, sneezing, and malaise, followed two days later by the passage of blood per vaginam immediately before the delivery of her baby by caesarean section. After reviewing the case it was considered that the infection had been transmitted through the placenta to the baby in the last days of pregnancy. When such congenital infection occurs, the baby may have signs of illness at birth or develop them soon afterwards.

It is not yet known whether Coxsackie Group B virus infections cause congenital malformations or death of the foetus in utero. This aspect of the problem needs further study.

Presumably mothers who are immune as a result of a previous infection will pass on their immunity passively to their offspring. Presumably, too, babies who are susceptible at birth have been born of mothers lacking the corresponding antibodies.

Clinical Features

Incubation period. The incubation period may be assessed from the ages of the infants at the time of onset of their illness. In the maternity home in Johannesburg this varied from 5 to 17 days. In the outbreak in Southern Rhodesia two of the babies were 4 and 8 days old respectively. As their mothers had no immunity subsequently, their infections must have been acquired after birth The incubation period of the illness in the one baby was 4 days or less and in the other, 8 days or less.

In the 4 cases in Holland the ages at onset were respectively 7, 8, 21, and 7 days. It may be deduced that the age at onset is frequently about 7 days and that the incubation period is frequently less than 7 days and may be less than 4 days.

Course of illness. In some cases the illness showed a tendency to a diphasic course. The first phase was relatively mild and would have passed unrecognized but for the fact that the babies were having their temperatures taken at regular intervals. In addition to fever lasting one to three days, it was noted that the baby went off its feed, some developed coryza and others had loose stools containing mucus The first phase was followed by apparent recovery for one

In 1956 KIBRICK AND BENIRSCHKE reported their findings in a study carried out in 1954 of a case in a newborn infant, who died seven days after birth by caesarean section. At birth the condition of this infant appeared good, but several hours later she developed a croupy cough. On the 3rd day her rectal temperature rose to 100.2°F. On the 4th day she was afebrile, but on the 5th day the fever recurred and a lumbar puncture revealed the presence of a pleocytosis and an increased protein content. On the 6th day her condition abruptly grew worse and she collapsed and died on the 7th day.

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In the outbreak reported from Southern Rhodesia and in the cases reported from Holland, the mothers or some other member of the family had suffered from a short pyrexial illness shortly before

ing of the fontanelle, neck stiffness, and a pleocytosis in the cerebrospinal fluid Jaundice was noted in several cases.

Laboratory tests. Routine laboratory tests were done on most of the reported cases, but revealed nothing of unusual significance. Microscopic and chemical examination of the urine showed no abnormalities Blood counts showed a normal or slightly increased total leucocyte count. Blood cultures for bacteria remained sterile.

Outcome of illness In the cases who recovered the fever subsided usually within a week, the condition of the infants improved rapidly and they were discharged from hospital apparently normal two to four weeks after admission. Several cases have now been followed up for four years and have shown no evidence of permanent cardiac damage

In the fatal cases, the patient's condition deteriorated with extraordinary rapidity, the complexion became an ashen grey colour, the temperature often dropped as circulatory collapse progressed, and death took place sometimes within a few hours of the apparent onset or within a few hours of the recrudescence of the fever.

Pathological Findings

Postmortem studies of several cases, notably those carried out by PEPLER AND LURIE in the first outbreak in Johannesburg, have given a clear picture of the pathological features

Macroscopic Appearances The infants usually have appeared well nourished and have shown no external evidence of disease, injury, or congenital abnormalities.

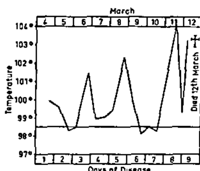
The peritoneum and sac have shown some oedema, but no other significant abnormality. The pleura has shown few petechial haemorrhages, occasionally an area of lack of lustre, and a slight fibrinous exudate.

The pericardial sac often contained an excess of fluid and occasionally has shown petechial haemorrhages.

The heart in some cases has been almost normal in macroscopic appearance In most it has been noted that it was slightly enlarged with dilatation of both auricles and ventricles, the muscle was flabby, somewhat thickened, and showed areas of pale mottling

The upper respiratory tract has usually appeared normal, occasionally was congested and contained mucus. The lungs have shown marked congestion and areas of atelectasis.

to seven days, then the severe illness of the second phase began. In some of the reported cases this biphasic course was not observed and the baby was desperately ill from the apparent onset. This severe phase of the illness was characterized by fever, a rapid grunting respiration, and a rapid pulse, followed by cyanosis of the lips, and a dusky mottling of the skin to be succeeded by the grey ashen pallor of circulatory collapse.



The heart rate was rapid and often the tachycardia was extreme with rates of over 200 beats per minute. At first there was no enlargement and the sounds were normal. Later signs of enlargement appeared. Arrhythmia and gallop rhythm also were noted. Signs of failure became apparent. In the chest there were signs of congestion, oedema and atelectasis, the abdomen was often slightly distended and in some cases enlargements of the liver and spleen were detected. Some cases also developed oedema of the legs and back. The systolic blood pressure also showed a marked fall.

Electrocardiographic changes. Electrocardiograms have been prepared in several cases. One of the cases in Holland showed inverted QRS complexes in lead I, with flattening of T waves in leads I, II, AV and V3 and V6. In V5 and V6 the R tops were absent and the ST segments were elevated. VAN CREVELD AND DE JAGER have noted that such an electrocardiogram shows a marked resemblance to that of an infarction of the anterior wall of the heart. In other cases prolonged P-R interval and flattening of T1 have been noted. These changes are indicative of myocarditis.

In several of the babies signs of involvement of the central nervous system also developed. These included drowsiness, stupor bulg-



corpuscles have been increased in size, and in many a central necrosis has been found with pyknosis and fragmentation of the cell nuclei associated with a proliferation of the reticulum cells.

Similar changes were noted in the follicles of some lymph glands which also showed marked congestion, and sometimes haemorrhages. The thymus thyroid and parathyroid glands and the pancreas showed congestion only.

The voluntary muscles and brown fat have not yet been adequately examined, but small fragments attached to other tissues did not show any pathological changes.

Of the abdominal organs the liver, spleen, and kidneys have shown marked congestion and some enlargement. The suprarenal also has shown marked congestion and in one case gross haemorrhage.

Microscopic Findings. The outstanding features were found in the *heart. The pericardium occasionally showed some inflammatory cells* The endocardium often showed some thickening and oedema and a *few focal infiltrations of inflammatory cells including lymphocytes* and a few polymorphonuclear and eosinophil leucocytes. The myo*cardium showed some congestion and a most striking pleomorphic* cellular infiltrate, which was often patchy and focal, occasionally more diffuse The foci were not sharply circumscribed but faded into relatively normal cardiac muscle. In the foci the muscle showed loss *of striation, swelling and eosinophilic degeneration and often frag*mentation associated with an infiltration of inflammatory cells consisting of lymphocytes, mononuclear cells, reticulum cells, histocytes, plasma cells and polymorphonuclear and eosinophil leucocytes. In these areas of inflammation, cells with pyknotic nuclei or pyknotic fragments of nuclei were frequently seen. Occasionally basophilic granules, somewhat suggestive of inclusion bodies, but possibly ingested nuclear fragments were seen in the cytoplasm of histocytes.

In some cases the pleura has been found to be thickened and oedematous and to be infiltrated with inflammatory cells. The surface may be covered by a thin layer of fibrin containing inflammatory cells.

The lungs always have shown marked congestion and dilatation of the alveolar capillaries, often associated with the extravasation of blood and oedema fluid into the alveoli

The liver usually has shown marked congestion and dilatation of the sinusoids and central vein sometimes associated with disappearance of the central parenchymal cells. The portal tracts have shown *an infiltration of inflammatory cells. Occasionally in addition there* have been foci of inflammatory cells, including lymphocytes, neutro*phil and eosinophil leucocytes.*

The kidneys have shown congestion of the glomeruli and inter*stitial tissue, and in one case an interstitial small focus of inflamma*tion consisting of lymphocytes and some eosinophil leucocytes.

The suprarenal glands have always exhibited marked congestion [illegible]

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corpuscles have been increased in size, and in many a central necrosis has been found with pyknosis and fragmentation of the cell nuclei associated with a proliferation of the reticulum cells

Similar changes were noted in the follicles of some lymph glands which also showed marked congestion, and sometimes haemorrhages. The thymus thyroid and parathyroid glands and the pancreas showed congestion only.

The voluntary muscles and brown fat have not yet been adequately examined, but small fragments attached to other tissues did not show any pathological changes

Of the abdominal organs the liver, spleen, and kidneys have shown marked congestion and some enlargement. The suprarenal also has shown marked congestion and in one case gross haemorrhage.

Microscopic Findings. The outstanding features were found in the heart. The pericardium occasionally showed some inflammatory cells. The endocardium often showed some thickening and oedema and a few focal infiltrations of inflammatory cells including lymphocytes and a few polymorphonuclear and eosinophil leucocytes. The myocardium showed some congestion and a most striking pleomorphic cellular infiltrate, which was often patchy and focal, occasionally more diffuse. The foci were not sharply circumscribed but faded into relatively normal cardiac muscle. In the foci the muscle showed loss of striation, swelling and eosinophilic degeneration and often fragmentation associated with an infiltration of inflammatory cells consisting of lymphocytes, mononuclear cells, reticulum cells, histocytes, plasma cells and polymorphonuclear and eosinophil leucocytes. In these areas of inflammation, cells with pyknotic nuclei or pyknotic fragments of nuclei were frequently seen. Occasionally basophilic granules, somewhat suggestive of inclusion bodies, but possibly ingested nuclear fragments were seen in the cytoplasm of histocytes.

In some cases the pleura has been found to be thickened and oedematous and to be infiltrated with inflammatory cells. The surface may be covered by a thin layer of fibrin containing inflammatory cells.

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The kidneys have shown congestion of the glomeruli and interstitial tissue, and in one case an interstitial small focus of inflammation consisting of lymphocytes and some eosinophil leucocytes.

The suprarenal glands have always exhibited marked congestion and in one case a gross haemorrhage, and in another a few small foci of inflammatory cells, mostly of the mononuclear type in the medulla.

The spleen has usually been enlarged and congested, and occasionally small haemorrhages have been seen in the pulp. The Malpighian



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Lesions of the brain and spinal cord were noted in several cases. The meninges were congested and oedematous and occasionally showed a slight diffuse infiltration with inflammatory cells. The brain substance usually were congested and often petechial haemorrhages were seen Focal collections of inflammatory cells, often associated with nuclear debris, have been noticed in the substance of the brain and in relation to blood vessels. The endothelium of these vessels was often swollen

In the case described by KIBRICK AND BENIRSCHKE, in addition to typical myocarditis, extensive involvement of the central nervous system was found. Lesions occurred in the spinal cord, pons, and cerebellum, in which there was destruction of some nerve tracts, degeneration of ganglion cells associated with an infiltration of macrophages, and polymorphonuclear leucocytes.

Virological Studies

In each of the outbreaks described there has been clear evidence incriminating *Coxsackie Group B viruses as the aetiological agent.* Convincing confirmation of their role has been obtained in several of the recent cases by the isolation of this virus from the heart muscle and from the tissues of the central nervous system.

Isolation of virus. Coxsackie Group B *type 3 virus was isolated* from the faeces of one of the patients in each of the first two outbreaks in South Africa. Baby mice inoculated with suspensions of brain from two fatal cases in the first outbreak exhibited lesions similar to those found in Coxsackie Group B infections.

Coxsackie Group B type 4 virus was isolated from the faeces of one of the two patients who recovered and from the faeces and caecal contents of the baby who died in the Southern Rhodesian outbreak.

The isolation of virus from the faeces does not necessarily incriminate it as the cause of the patients illness, nor does the demonstration of the subsequent development of immunity against this virus However, final proof of the relationship of Coxsackie virus to this condition has *been* provided *by its isolation from the heart muscle.* This was achieved in the case admitted to the Transvaal Memorial Hospital for Children and also in the four cases described by VAN CREVELD AND DE JAGER. Coxsackie Group B type 2 virus was iso-

lated from the heart muscle of the former case and Coxsackie Group B type 4 from the heart muscles of the latter 4 cases, and in one of them from the brain tissue as well. In Boston in the United States Coxsackie Group B type 3 virus was isolated from the spinal cord of the case described by KIBRICK AND BENIRSCHKE. More recently ENDERS has briefly noted the isolation of Coxsackie Group B type 4 virus from a similar case in the same city.

Types of Coxsackie Virus Concerned In the studies so far carried out, Coxsackie Group B types 2, 3 and 4 have been incriminated Types 1 and 5 have not yet been implicated, but this of course does not exclude them from being potential causes. Only future studies will reveal whether one type is more liable than others to cause this condition.

These viruses have been isolated by the inoculation of suitably prepared suspensions into baby mice. The virus has also been isolated directly in tissue culture The identity of the isolated virus has been established both in baby mice and in tissue culture protection tests, using type specific antisera prepared against viruses of known type.

The baby mice have shown lesions of the fat pad, in the early stages of which there is degeneration, necrosis, and acute inflammation, followed by calcification In addition to this unique lesion characteristic of Coxsackie B infection, lesions have also been found in the brain, in which degeneration and destruction of individual neurones with pyknosis and fragmentation of the cell nuclei have been observed. A perivascular cuffing with lymphocytes and monocytes of the blood vessels has been found in relation to such lesions. Softening and rarefaction of the white matter, and in some instances a considerable "fall out" of neurones best seen in the hippocampus has been noted There is usually an associated slight infiltration of inflammatory cells in the meninges The hearts of a number have showed foci of eosinophil degeneration and fragmentation and dissolution of the muscle fibres, pyknosis of the nuclei, and an infiltration of inflammatory cells. Occasionally other organs, notably the pancreas and liver, have shown similar focal lesions.

These lesions observed in baby mice are similar in character and distribution to those noted in the babies dying of this infection

Monkey Pathogenicity of Strains. VERLINDE and his co-workers tested the monkey pathogenicity of strains isolated in Holland. Two cynomolgus monkeys, each about 6 months old, were inoculated intracerebrally. Both developed fever and virus was recovered from

the blood. One of these monkeys developed a second peak of temperature on the fifth day. Neither showed clinical signs of illness.

One two-day old cynomolgus monkey was inoculated intramuscularly with a suspension prepared from the second suckling mouse passage of virus. The animal became severely ill on the second day, the main symptom being dyspnoea This animal was sacrificed and virus was demonstrated by the inoculation of suckling mice with suspensions prepared from the heart and brain. Histological examination revealed the presence of lesions in the heart muscle in which the myofibrils had lost their striation, the nuclei were irregularly shaped, and either hyperchromatic or pyknotic associated with a few dispersed leucocytes, but no marked cellular infiltration.

Preventive Measures

The possibility of preventing these cases and outbreaks has been discussed by GEAR (1957). He recommended that when Coxsackie virus infection is prevalent in the general population, precautions should be taken to prevent its introduction into maternity homes. Expectant mothers with a feverish illness or who are known to have been exposed recently to infection, should be admitted to a ward where it would be possible to isolate them and their babies from others. Staff members developing signs and symptoms of Bornholm disease or other suspicious illness should be put off duty for about one month. If an outbreak occurs in a maternity home, the affected ward should be closed temporarily to enable all the babies and their mothers to be evacuated. Before any new admissions are allowed the accommodation should be disinfected and the equipment sterilised. Pregnant women may be tested for their immunity to the five serotypes of Coxsackie B virus The immunization of those lacking antibody to the prevalent type of virus may be feasible However, the value of prophylactic vaccination of prospective mothers and of the administration of gamma globulin in protecting babies exposed to infection has not yet been assessed.

Discussion

Editorials in the New England Journal of Medicine (November 8th, 1956) and BLATTNER in comments on current literature in the Journal of Pediatrics (May 1957) in reviewing the reports of the above

described outbreaks, have called attention to the increasing importance of myocarditis in infants and it has been suggested that the condition should be termed "acute aseptic myocarditis".

The epidemiology of Coxsackie virus infections has many resemblances to that of poliomyelitis. With improvement of standards of living and hygiene, a similar increasing incidence of overt manifestations of infection may be expected. As the proportion of adults who have escaped earlier infection increases so will there be a corresponding increase in the number of babies lacking protection passively acquired from their mothers. With this will go a corresponding increase in the number of babies liable to encephalo-myocarditis.

However, although a problem of increasing concern, myocarditis has been recognized as one of the hazards of infancy for many years. SAPHIR in 1941 gave a general review of the subject. Since that time, judging from the reports in the literature, it appears to have increased in incidence. Series of cases have been reported by SAPHIR, WILE AND REINGOLD (1944), by KELLER (1945), by LIND AND HULTQUIST (1949) and by WILLIAMS, O'REILLY AND WILLIAMS (1953) and by DRENNAN (1953). BOWDEN AND FRENCH (1951) have also called attention to the importance of acute myocarditis as a cause of unexpected deaths in children.

In 1952 STOEBER reported on the pathological findings in 140 autopsied cases of epidemic myocarditis of infancy. Clinically the cases were characterized by loss of appetite, by vomiting, cyanosis, dyspnoea, tachycardia, hepatomegaly, and occasionally splenomegaly The illness was of short duration in those who survived. At postmortem cardiac dilatation and some thickening of the muscle were seen. This was often associated with broncho-pneumonia, tracheobronchitis, and hepatomegaly. Histological examination of the hearts revealed myocarditis, with a picture similar to that seen in myocarditis of the newborn due to Coxsackie Group B virus. However, the peak incidence of these cases occurred at one year of age.

SAPHIR AND COHEN (1957) have again reviewed the literature concerning this condition and have given an account of five new cases of their own. These five infants, two of whom were 7 days old, one 11 days, and one 9 months, and one 12 months, died after a very short illness Evidence of myocardial changes was found clinically and electrocardiographically in three cases. At autopsy myocarditis was found associated with necrosis of varying degree. of groups or of isolated muscle fibres

the blood. One of these monkeys developed a second peak of temperature on the fifth day. Neither showed clinical signs of illness.

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WILLIAMS, H, O'REILLY, R H, and WILLIAMS, A Fourteen cases of idiopathic myocarditis in infants and children Arch Dis Child. 28 271–283 (1953)

They also analyzed the age incidence of 90 cases of myocarditis reported since 1948, 47% were less than 6 months old, 71% were under 1 year of age. Only 12 died after the age of 18 months.

Although virus studies were not carried out in their cases, they believe that the histological features suggested that Coxsackie virus was probably the cause of the disease in their 2 seven-day old babies. It is an intriguing question not yet answered whether the cases in older infants and in children have a similar aetiology. It is a problem which certainly merits further and intensive investigation, the results of which will be awaited with great interest by all those concerned with the health of infants and children.

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achieved and these vaccines were withdrawn from use (1, 2). Further trials of experimental poliovirus vaccines were not undertaken until Enders, Weller and Robbins (3) in 1949 reported the successful growth of the virus in tissue culture. This finding, in addition to providing a readily available source of high titer virus, also provided an independent and sensitive means for the measurement of virus activity. Early in 1953 (4, 5) a process was presented which, based on hypothetical inactivation kinetics, was believed capable of producing consistently a safe and effective inactivated poliovirus vaccine. Plans were made for an extensive field trial of such vaccine by the National Foundation for Infantile Paralysis with the cooperation of pharmaceutical manufactures

The 1954 Field Trial was carried forward and evaluated as completely successful (6), and on April 12, 1955, the vaccine became a licensed product under United States Public Health Service regualtions. The pharmaceutical manufacturers were licensed immediately to distribute poliovirus vaccine produced under the tentative Minimum Requirements which had been established on May 20, 1954, almost a year prior to the licensing The licensing was on the basis of the manufacturing and testing experience obtained during the preparation of the 1954 Field Trial vaccines (supplied by 2 manufacturers), and 5 producers, who had independently adapted the original process to their facilities and manufacturing practices, entered the market

During the development and adaptation of the originally described laboratory process (5, 6) to the large-scale manufacturing operations, extensive experimental data were obtained which indicated the simple and inviting theoretical concept that inactivation of poliovirus with formaldehyde followed first order kinetics was open to question and revision (7–15). By May 26, 1955, it was evident that the experiences of the early 1930's had been repeated – a number of cases of poliomyelitis was definitely associated with the administration of vaccines, subsequently shown to contain residual living virus (10, 11, 12). As a result of this development, the Technical Committee on Poliomyelitis Vaccine was formed as a permanent poliovirus advisory group to the United States Public Health Service on the release of vaccine lots and to give guidance on vaccine production and testing. In a report (June, 1955) (13) the Committee reviewed the initial standards, manufacturing practices and testing procedures, and proposed measures to deal with the then current and also projec-

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From the Research Laboratories, Parke, Davis and Company
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nucleoprotein. The biological activities (infectiousness and antigenicity) undoubtedly require the intact particle for full expression of their capacities, but it was observed (16) that treatment with glycerol which appeared to remove the peripheral portion of the virus particles did not produce complete loss of the capacity of the virus to infect monkey tissue cells.

It has been shown repeatedly that the capacity of animal viruses to incite infection and virus multiplication resides in the virus nucleic acid portion, whereas certain specific antigenic properties of the virus seem to be associated with and are determined by the protein moiety. It has been suggested (30) that virus protein in the form of an outer coating affords protection for the more labile genetically specific nucleoprotein portion (multiplication initiation factor). The peripheral protein also contains the mechanism (receptors) whereby the viruses attach to cells and initiate infection. This concept is particularly attractive in the case of poliomyelitis virus (Figures 1 and 2).

Cultivation of Poliovirus in Tissue Culture

Following the demonstration (3) that poliovirus could be cultivated in tissue cultures of non-neural cells of anthropoid origin, a large amount of work in a number of laboratories indicated the practicability of this procedure for the preparation of large volumes of poliovirus antigen Much of the early work leading to the preparation of the first vaccines (4) was done in roller-tube cultures of minced simian or human tissue embedded in a chick plasma clot. This procedure was, however, soon superseded by more efficient means of producing the virus, and monkey kidney tissue became the standard source of cells (31, 32) The first method to be used on a large scale was developed by the group at Connaught Laboratories (31) and employed minced monkey kidney tissue maintained in suspended culture in rocking bottles Virus was grown by this method for preparation of the majority of the vaccine lots prepared for the field trials, and it is still used by some vaccine producers. An alternate method utilizing trypsin dispersion of the monkey kidney cells and cultivation of the dispersed cells as monolayers directly on glass was suggested by YOUNGNER (32) This later method gives, 1) greater virus yields per kidney used, 2) fluids with lower extraneous protein

ted long-range problems in the poliomyelitis vaccine production. To a large degree the changes and the revisions made were based empirically upon critical analyses of past manufacturing experiences under a method already suspect; subsequent experience, however, has shown that the vaccines produced and released during the next two years, following the revised *additional poliomyelitis vaccine standards* (14), were of acceptable safety and effectiveness.

A number of statistical and critical reviews of the procedures (8, 13, 15) have been published, but without the integration of certain *basic and fundamental information.* Further retrospective review, without critical consideration of certain aspects regarding the fundamental nature and properties of poliovirus itself, the dynamics of inactivation mechanisms involved, and the application and adaptation of these fundamentals to the technologic details of vaccine manufacturing, would seem futile and to a large degree meaningless. Therefore, the experimental data obtained in the processing of the vaccines for the National Foundation for Infantile Paralysis field trials, detailed studies of inactivation procedures, and the deductive integration of these data into a combination inactivation process and the associated testing programs have been the main theme of a series of publications in the Journal of Immunology from these laboratories (16–21). The purpose of this paper is to condense and present, as nearly as possible in chronological order of the factual developments, our experiences *in the production of poliovirus vaccines Necessarily, there will be* grouping of certain aspects under specific headings.

Poliovirus – Fundamental Properties

Early in 1953 purified poliovirus particles were obtained by concentration from tissue culture fluids. It was possible to describe the virus as a distinct particulate entity, approximately 30 mμ in overall diameter (16, 22, 23, 24), and composed of 22 to 30 per cent RNA (16, 25) probably contained in a central body approximately 20 mμ in diameter. This virus, like other animal viruses (26, 27, 28, 29), is comprised of an outer membranous structure surrounding a plasm which contains an internal nucleus-like body (16). The outer hydrated peripheral layer, which collapses or shrinks on drying is less electron-opaque and is probably largely protein in nature. The dense electron-opaque central core is most likely composed of the nucleic acid and/or

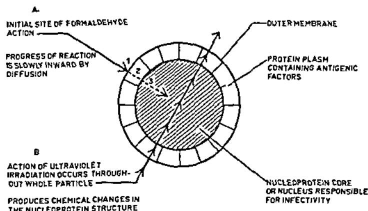


Fig 2 Schematic cross-section of poliomyelitis virus particle showing differences in the concepts of the action of formaldehyde and ultraviolet irradiation

content; 3) better control of extraneous viruses derived from the tissue source, and 4) greater flexibility in controlling production schedules. For these reasons several manufacturers used this method for cultivating poliovirus in the processing of vaccine. One of the vaccine lots giving good antibody response in the field trial (6) was made from virus grown in this manner. However, one difficulty encountered with the trypsin-cell-produced virus was excessive loss of virus activity when filtered by the asbestos pad method developed with the "Connaught-type" fluids. Finely dispersed cell debris tended to work into and plug the filter pads. Initially, this problem was overcome by changing to fritted glass filtration, but it is now known that the more adequate and generally accepted "Seitz" filtration can be utilized under proper conditions. The most important factors are the storage conditions; namely, the period of time the fluid is held before treatment and the method of preliminary clarification used.

The use of other monkey tissues and of human or simian cells maintained in continuous culture has not been proven to be practical on a production scale. It is not unlikely, however, that future developments may result in improved methods of growing poliovirus suitable for the preparation of vaccines.

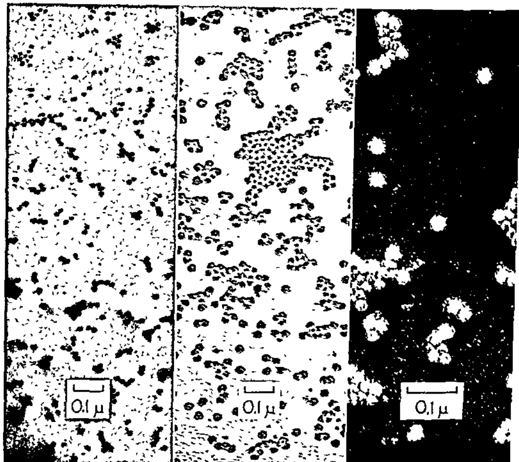


Fig 1 Left Poliovirus, Type III (Saukett) unshadowed The transmission image is ½ to ⅔ the size of the shadowed image Definite spacing is observable in the closely-packed arrays, and the dense electron-opaque image suggests that it is an internal nuclear-like structure 85000 X. *Center* Poliovirus, Type II (MEF-1), practically unshadowed. A trace of chromium was evaporated on the specimen in *order to render the peripheral portions of the particles sufficiently opaque ot be* imaged by the electron beam 85000 X. *Right* Poliovirus, Type II (MEF-1), prepa- [illegible] lightly shadowed with chro-

[illegible]

an extensive study (TAYLOR and McCORMICK, Yale J Biol Med, 28 [illegible] (1956) of the morphology of poliovirus which led to the hypothesis that the characteristic, approximately 30 mμ diameter, particles of poliovirus are "cell-like", containing a central body with a peripheral plasm surrounded by a *limiting membrane*

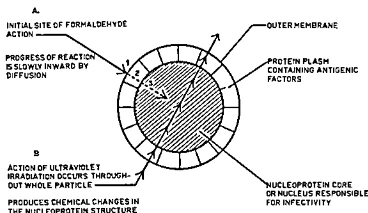


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Inactivation of Poliovirus

A. Formaldehyde

1. Nature of Formaldehyde Reaction with Organic Material.

Formaldehyde is a typical tanning agent and has been used for many years to follow protein degradation by the quantitative determination of amino acids (33). The kinetics of formol reactions are complex and of great theoretical significance, but the picture, in relation to the actual kinetics of the reactions involved, is far from complete (33, 34). Amino and imino groups appear to be the most reactive, but, in addition, peptide linkages, ring structures, sulfhydryl and hydroxyl groups will react. Most formaldehyde reactions with proteins proceed in 2 or more stages, the initial stage being fully reservible, while the later secondary reactions are irreversible. An initial equilibrium between free and reversibly bound formaldehyde occurs, followed by a gradual disappearance of chemically demonstrable free formaldehyde. The ensuing secondary reactions are characterized by methylene and polymethylene bridging between sites of initial reaction with the emergence of complex ring structures. Thus a firmer, less flexible structure of the protein acted upon is evolved by the gradual saturation of charged and reactive groups. The protein *is rendered less soluble and chemically (and biologically) more inert.* It is possible to follow and analyze these reactions with some degree of success only when a relatively pure material is being dealt with and conditions can be controlled. Any interference with such a highly labile system is apt to disturb delicate equilibrium conditions and thus produce a distorted picture Certain factors must be controllable at least to a degree; namely, concentration of material acted upon, concentration of formaldehyde, pH, temperature and presence of extraneous material—to name but a few In summary it may be said that when formaldehyde and a protein interact, stepwise reaction appears to be the rule and over-all linearity the exception.

2. Theoretical Aspects of Virus Inactivation by Formaldehyde.

Under ideal conditions when a purified poliovirus suspension of known concentration in a buffer system where the formaldehyde concentration, pH, temperature, etc., are all controllable, the re-

action between the virus and formaldehyde could conceivably be analyzed and some degree of reproducibility and predictability expected. Due to the complexity of the virus structure itself, however, the reaction would not be expected to proceed strictly according to first order kinetics, but rather at least 2 distinct general effects, possibly more, would be anticipated. First, a reaction would occur with the protein coat material which presumably would be rather rapid. Such is actually the case (7, 35, 36, 37); treatment of poliovirus with 1:4000 formaldehyde produces an immediately apparent, rapidly progressing loss of infectious capacity due either to the blocking of receptor sites, or to a hardening or tanning of the surface of the virus particle. This could interfere with the release of the nucleic acid and initiation of virus multiplication in susceptible cultures (20) Such a reaction, proceeding mainly on the surface of the virus particle (Figure 2A), would follow pseudo-first order reaction kinetics (7) as long as the virus suspension were homogeneous and the medium contained an excess of free formaldehyde Secondly, there would be occurring simultaneously an irreversible chemical alteration, of the protein and the vital nucleic acid of the virus which would lead eventually to complete destruction of the capacity for multiplication (true inactivation). This reaction also would be pseudo-first order in the presence of excess formaldehyde, but would occur at a slower rate Therefore, the final result would entail in our idealized situation at least 2, possibly 3 or more, steps The outer protein coat would have to be saturated completely with formaldehyde, the formaldehyde then would have to diffuse through the hydrated protein layer and react with the nucleoprotein to render it nonreproductive. Thus, even with a purified virus suspension where there are obviously at least 2 general sites of competition for the formaldehyde within the virus particle itself (Figure 2), the analysis could be expected at best to be only the summation of a series of pseudo-first order reactions. In the actual very complex situation the virus, not necessarily monodisperse, is suspended in tissue culture fluid where the medium constituents, amino acids, growth substances, and monkey kidney cell protein are all in variable concentration, and are all competing for the available formaldehyde. In this situation the analysis of the kinetics, which are very involved, would be further complicated. Certain essential factors of this complexity have been elucidated by the work of GARD (35), HAAS ET AL. (36), WESSLEN ET AL. (37), LYCKE ET AL. (38) and others (7, 20, 39). There are, in addition,

certain difficulties in later stages of the reaction, the "so-called" tailing effects due to aggregation or other factors. This will be discussed in more detail below. However, in summary it may be stated that the demonstration *of an apparent straight-line inactivation* over a limited portion of the reaction is theoretically possible (4, 9). But such a demonstration is dependent upon chance selection of conditions and the data so obtained are not adequate for practical application in controlling vaccine production (7, 19, 20).

3. *The Nature of Formaldehyde Inactivation of Poliovirus.*

During the production of the earliest commercial vaccines for the 1954 Field Trials, it was observed in this laboratory that formalin-treated suspensions of virus appeared to have different characteristics of growth in tissue culture than nontreated suspensions (7, 18, 19, 20). Figure 3 is reproduced from the official report on the processing of the first commercial lot of poliovirus vaccine (20). These fluids (1 pool of each virus type) had been prepared on Maitland-type cultures. They were treated in *December, 1953, with 1:4000 formalin following the* recommended procedure (5, 40). Samples removed from the bulk pool at 24-hour intervals were titrated in 15 minced monkey testicular

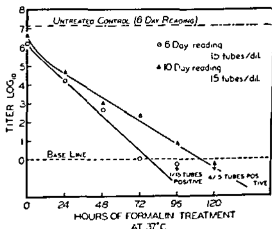


Fig 3. Formaldehyde inactivation rate test of the Mahoney strain, Type I, component of Connaught Lot No 1 J Immunol. (20)

tube cultures per tenfold dilution. The cultures were incubated at 37°C. and examined daily, and the cytopathogenic endpoint titers determined from 6- and 10-day readings of the tubes. Only the data for pool C-No. 1 (Type I, Mahoney strain) are shown. Similar results were obtained on parallel tests of the MEF-1 and Saukett strains. The titers estimated for the treated samples from the 6th day readings are lower than those for the readings 4 days later due to the continuing development of positive tubes. No increase was observed in titer or late appearance of positives with the nonformalinized control tests The apparent increase in titer of the rate samples becomes greater as the time of formalin treatment progresses; in other words, there was a delay in appearance of positive cultures in formaldehyde-treated virus.

The formaldehyde inactivation process (4, 5, 9, 13, 14, 40, 41) was assumed to have a margin of safety which was achieved by measuring the rate of inactivation, during the first 72 to 96 hours, then adding a calculated time increment of additional formalin treatment. But, since the minimum inactivation time as estimated by extrapolation of the rate line is dependent upon the period of observation of the test cultures, this method of estimating complete inactivation could not be relied upon. Also, this finding proved that a prolonged observation period of the tissue culture safety test cultures was not only indicated, but imperative—the tissue culture safety test being the only valid control of the inactivation process.

Extension of the tissue culture safety test eventually to a total of 35 days of observation (19) was immediately instituted in these laboratories (February 1, 1954) and a detailed integrated study of the formalin inactivation procedure was undertaken (7). Data were collected from production rate curves and also from specific rate experiments in which all of the potential variables were changed one at a time to permit detailed study of the effect of each variable upon the reaction These changes included formalin concentration, temperature, pH, and media alterations Experiments were included in which various degrees and types of filtration were employed to permit evaluation of the degree of clarity or "*monodispersity*" *of the virus* suspensions upon the rate and course of the inactivation pattern All samples and controls were run in duplicate or triplicate to ensure that the data obtained represented the true pattern of the reaction and could be used as a factual basis for the development of large-scale manufacturing techniques

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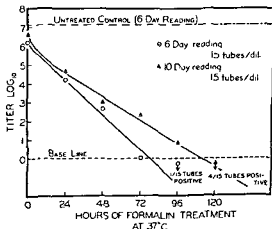


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It was found (7) that during the first few hours of formaldehyde treatment there is apparently a sharp drop in infectious titer of the virus which is then followed for the ensuing 48 to 120 hours (*Figure 3*) by an apparently linear, progressive loss in titer at a slower rate than that of the initial decline (7). This pattern persists regardless of the experimental conditions, even though the absolute rate of the reaction can be varied by changes in pH and temperature, and in formalin *and extraneous* protein concentration.

It was pointed out (7, 18, 19, 20) that the reaction could not be treated as first order, even if the "tailing" effects observed in the later stages of the inactivation are not considered. This very real problem has been discussed recently in detail by GARD (33) and cannot be minimized It has been shown that there is a progressive increase in light scattering due to an increase in suspended particulates in tissue culture suspensions of poliovirus treated with formaldehyde (39, 42). This aggregation due to protein denaturation *or* formaldehyde bridging (34, 36) *could well result in the incorporation and protection of* trace amounts of virus. It is *more important* perhaps to filter immediately after the addition of the formaldehyde and the result of an experiment carried out in early 1955 demonstrating this is summarized:

Unfiltered Type I virus was divided. One-half (Vaccine A) was filtered through a series of coarse, medium, fine and double ultra-fine sintered glass candles, adjusted to pH 7.0 and 1·4000 formalinized. The other half (Vaccine B) was formalinized before filtration through a similar series of candles and then pH adjusted to 7.0. Both vaccines were incubated at 37°C. Starting at 5 days, samples were removed and the results of comparative safety tests (a total of 500 ml. per test in 10 monkey kidney cell culture bottles) (19) are shown in Table I. The slightly lower total formaldehyde content of Vaccine B might indicate that there was a slight loss on the cell debris removed by the filtration.

It is noteworthy that trace amounts of active virus were still found in Vaccine A after 17 days of incubation with formaldehyde, whereas inactivation was complete after 9 days of incubation in Vaccine B. The safety test bottles were observed daily and the time *that a cytopathogenic effect was first* noted in each test is recorded. As will be discussed subsequently, the trace quantities of virus remaining after formalin treatment are usually detected in the 3rd week of the safety test. It is probable that these results, at least in part, are due to clumping of the virus particles. Freshly filtered

Table I Effect of filtration immediately following the addition of formaldehyde upon total time required for complete inactivation of poliovirus

Time Tested After Treatment	Vaccine A Filtered Before Formaldehyde Added	Vaccine B Filtered After Formaldehyde Added
5 Days	10/10 (7 Days) *	8/10 (16 Days)
7 Days	10/10 (10 Days)	3/10 (18 Days)
9 Days	8/10 (7 Days)	0/10
11 Days	1/10 (21 Days)	0/10
13 Days	5/10 (16 Days)	0/10
15 Days	0/10	0/10
17 Days**	1/10 (18 Days)	0/10

* Ten culture bottles were inoculated with each sample numerator of fraction indicates number of bottles positive Day of first appearance of positive cultures in each test is indicated in parenthesis

** Vaccine A contained 120 gamma and Vaccine B, 108 gamma of total formaldehyde at the end of 17 days of treatment

material contains very sizeable aggregates, Figure 4 However, if formalin is present during the filtration, the microscopic, but nevertheless violent, shearing effects and agitation inherent in the filtration process result in the breaking up or filtering out of clumps, and also achieve a more effective exposure of the virus particles to the formaldehyde

Similar "tailing" effects, but of different and usually lesser degrees, have been observed with all inactivation procedures studied (heat, ultraviolet, as well as other chemical treatments): various explanations of this, such as *nonuniformity of populations*, multiplicity reactivation, and the like, should not be excluded without careful consideration.

4 Formaldehyde Inactivation Compared with Inactivation by Other Chemical and Physical Agents.

The effect of other chemical and physical agents upon poliovirus compared with the effect of formaldehyde revealed fundamental information of the greatest importance with respect to the practical

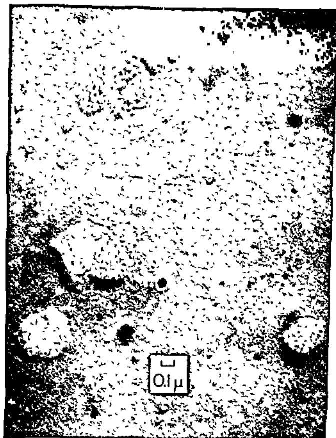


Fig 4 Electron micrograph of centrifugal clarifier residue (18) of freshly filtered poliovirus tissue culture fluid This illustrates the fact that filtered suspensions still contain aggregates and particulate material in the size range of rickettsiae *44 000 X*

use of formaldehyde as an inactivating agent. In an attempt to further clarify and define the mechanics of the inactivation, poliovirus was partially inactivated (20) by various chemical and physical agents; *formalin (1:4000 dilution of 37% formaldehyde for 24 hours at 37°C.,* then neutralized); ultraviolet irradiation (18 incident watts, 600 ml. per minute); iodine (I_2, 25 ppm for 10 minutes, then neutralized); beta-propiolactone (0 02% for 2 hours at 37°C.); heat (56°C. for 16 hours). The plaque titration test modified by HSUING and MELNICK (43) was used since it permitted a more quantitative measurement of the amount of infectious virus and its rate of appearance. The

percentages of total plaques appearing at daily intervals were used to compare the rate of initiation of infection by the variously treated materials. It had been noted earlier (7, 18) that with formalin-treated virus a lag phase occurred before infection of cells by residual virus which was more pronounced than the delay required for infection by diluted untreated virus or that treated by ultraviolet irradiation. Figure 5 is a bar graph showing the comparative rates of plaque appearance with the different types of inactivation. In all but the formalin-treated preparations the majority (80 to 90%) of the plaques had developed by the 3rd day. In contrast, only 0 to 13% of the total plaques had developed by the 3rd day and only 25 to 50% by the 4th day in the formalin-treated poliovirus tests These results show that formalin is unique among the agents studied in producing inactivation in that with the formalin-treated virus alone there was a marked delay in the appearance of plaques when the variously treated viruses were tested in parallel at the same time and on the same lot of cultures

Various factors were studied to determine, if possible, why this delay phenomenon occurs only with the formaldehyde-treated virus. The time of treatment with formaldehyde progressively increased the delay; it was most pronounced with Type I, somewhat less with Types II

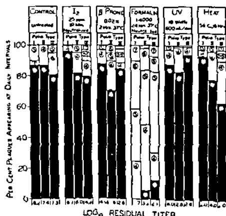


Fig 5 Rate of plaque appearance by polioviruses treated with various chemical

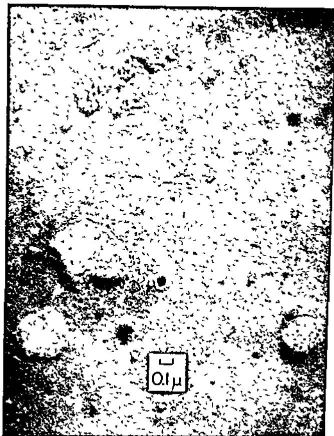


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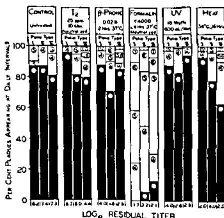


Fig 5 Rate of plaque appearance by polioviruses treated with various chemical and physical agents. Each bar is marked to indicate the cumulative percentages of plaques appearing after incubation periods of 3 to 7 days, the designated day is encircled. Early plaque development areas (3 days) are shaded. J Immunol (20)

and III in that order, but still highly significant in degree. When the effect of formalin concentration was incorporated into these experiments, the total extent of inactivation appeared to be the important factor in relation to the delay phenomenon. The inactivation can be produced more rapidly with increased formalin concentration, but the delay in initiation of infection increases progressively as the inactivation treatment progresses. If poliovirus is treated with formalin, then the formaldehyde neutralized or removed by dialysis, the delayed effect may be reserved to a degree. In retests, after storage for 1 week, the delay was less evident and the increased final titers obtained (20) indicated possible recovery of the ability of the residual virus to initiate infection. This trend was even more pronounced when the samples were again retested after a 12-week period of storage. The method utilized for the removal of excess formaldehyde did not affect the delayed rate of appearance of plaques, but if the buffer effect in the medium used in the test bottles was increased by the use of added bicarbonate and/or calf serum, an increased rate of plaque appearance was observed However, in spite of the more rapid initiation of infection, the final titers (PFU/ml.) attained were not increased. Therefore, the more rapid appearance of plaques was not caused by an increase in the number of infectious units but only in their rate of appearance. The earlier appearance of positives in the so-called high pH safety test devised by Hampil (44) and studied extensively by all of the manufacturers was quite probably another aspect of this phenomenon. The average size and over-all rate of growth of the plaques was the same for treated and untreated virus regardless of the concentration of, or the presence or absence of, serum or bicarbonate. This indicates that the decrease in the delay time of plaque appearance was not due to an absolute increase in rate of virus growth. Further, there was no difference in the rate of growth of early- or late-appearing plaques indicating that formalin treatment as such shows no evidence of a selection of genetic variants.

This prolongation of the latent period required for the initiation of cellular infection by formaldehyde treatment indicates a modified "avidity" of the treated virus for cells. Two possible mechanisms were suggested (20), a deficiency in ability to adsorb onto cells, or a delay in initiation of infection following the attachment. Also, the lowered "avidity" may be associated with an initial weaker virus-cell bond and the effect could be considered as due to an increase in the "dissociation constant" of the virus-cell complex.

5. Integration of Formaldehyde Inactivation Information

In actual practice it would appear that inactivation of poliovirus by formaldehyde could be pictured as a 3-step sequence of related reactions as shown in Figure 6. The reversible 1st stage, A, of rapid virus-formaldehyde complex formation is responsible for the initial rapid apparent loss of activity (7) and, if this reaction could be projected to "base line" levels, it would be complete in a relatively short period of time. This stage is triggered by heat (37° C.) since it is not demonstrable when formalin is added to virus-fluid at refrigerator temperature. However, if the fluid is at 37°C. when the addition of formaldehyde is made, the initial drop in titer of virus occurs even though the mixture is rapidly chilled, but such apparently inactive virus could be mistaken for completely noninfectious virus in short-term tests for activity (i e., rate tests). Although this reaction conceivably should be near completion within a few hours for the majority of the virus population in a suspension, actually this initial stage, A, progresses in a matter of 2 to 6 hours into the 2nd stage, B, where the initial relatively loose virus-formaldehyde combination

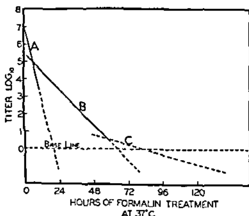


Fig. 6 Schematic representation of formaldahyde inactivation of poliovirus. A, first rapid virus-formaldehyde complex formation at least partially reversible B, second firm complex formation which occurs at a slower rate than A A combination of A and B under certain conditions has been used to obtain a "base line" intercept, and to predict inactivation time (see text) C, represents the so-called "tailing" effect due to aggregation, tanning, resistant virus ("masked" or "occluded" virus).

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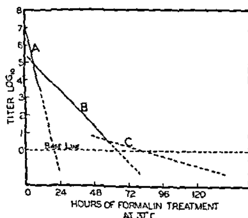


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progressively becomes a more firm union. If this stage were to be projected, as has been the practice, it would indicate a "base line" intercept or zero titer within say 48 to 96 hours depending upon the time of the selection of the "rate-samples". *Such a progressive mixed* reaction situation is not unique in the inactivation or alteration of biological systems and a great deal of additional data will be required before conclusions can be drawn concerning the actual kinetics involved. More important in a practical sense is the fact that the formaldehyde inactivation reaction is not simply "first order", and this is apparent from data obtainable during the first 48 to 72 hours of the reaction where the analyses are straightforward.

The possible explanations for the apparent difficulty in inactivating the last traces of viable virus with formaldehyde in a suspension are myriad and, since quantitative studies in this area are difficult to design, much speculation exists. This speculative 3rd stage, C in Figure 6, is represented by the lag in inactivation of the very small proportion of "resistant" or "masked" virus particles. In our experience prolonged incubation with formaldehyde is required to produce completely inactive virus (up to 21 days in some cases). It might be postulated that, whatever the protective mechanism is that produces the "tailing" effect in the pattern, the last stages of formaldehyde inactivation procedure are completed by thermal effects rather than by the formaldehyde. In other words, formaldehyde inactivation alone as utilized is in reality a combination procedure. Evidence from heat inactivation studies indicates that variation in the virus population in susceptibility to elevated temperature is quite marked (45). Clumping or aggregation of virus particles or protection of virus by cellular debris is almost surely a contributing factor in the "tailing" of the formaldehyde "rate curve". Aggregation may be directly induced by the formalin treatment (39, 42), or some few virus particles, and the protein cell debris may be tanned or fixed by formalin, thereby rendering the virus incapable of infecting the cell until "unmasked" by enzymatic action in tissue. That is, a small proportion of virus may not actually be inactivated in a true sense by formaldehyde. However, lack of monodispersity is not entirely responsible for the "tailing" effect and the importance of resistant genetic variants, reactivation by reversal or recombination, or other unknown factors remain to be assessed.

The unique cytopathogenic delay effects observed with formaldehyde-treated virus, and the fact that formaldehyde inactivation

proceeds as a sequence of reactions at varying rates rather than as a single "first order" type of reaction, would indicate that the "projection" of the inactivation "rate-curve" cannot be relied upon to estimate complete inactivation of poliovirus.

B. Ultraviolet

1. *Inactivation Effect of Ultraviolet Irradiation on Poliovirus*

Concurrently with the detailed study of formalin inactivation during 1953 and 1954, the application of ultraviolet irradiation as a method of inactivation was being investigated (17, 18). Inactivation rate studies were made upon the 3 strains of poliovirus in the form of freshly harvested infectious tissue culture fluids (medium 199) from trypsinized monkey kidney cultures. The fluids were filtered immediately prior to irradiation. Inactivation rate and ultraviolet absorption spectrum studies were made on virus concentrated (20×) by cold alcohol precipitation.

The centrifugal filmer ("Centri-Filmer") (46), developed by Oppenheimer and Levison (47) and engineered by the General Motors Research Staff (Special Problems Department), provided the basic instrument in which the irradiations were carried out. Developmental and operational details of the instrument as well as the methods of calculating the effective incident levels of ultraviolet energy used, are given in previous publications (17, 18, 48).

The primary component of the filmer is a vertical 15-inch stainless steel bowl (Figure 7) which rotates at a fixed speed (1750 rpm.). The inner walls of the bowl are inclined outward at a 1-degree angle so that fluid fed onto the bottom of the rotating bowl is centrifugally spread into an extremely thin film as it flows upward due to centrifugal force. In operation, 6 ultraviolet lamps (General Electric G18T6) in a special water-cooled holder (30° C.), are suspended inside the rotating bowl so that approximately a 750 sq. cm. area of the inside surface of the bowl with its flowing thin film of fluid receives incident ultraviolet energy of controllable intensity. The effective incident ultraviolet energy produced by the lamps can be varied from approximately 5 watts to over 30 watts by varying the number of lamps used and the input voltage and current to the lamps. This, in turn, can be combined with different flow rates of the fluid being irradiated to provide various degrees of ultraviolet exposure. The

progressively becomes a more firm union. If this stage were to be projected, as has been the practice, it would indicate a "base line" intercept or zero titer within say 48 to 96 hours depending upon the time of the selection of the "rate-samples". Such a progressive mixed reaction situation is not unique in the inactivation or alteration of biological systems and a great deal of additional data will be required before conclusions can be drawn concerning the actual kinetics involved. More important in a practical sense is the fact that the formaldehyde inactivation reaction is not simply "first order", and this is apparent from data obtainable during the first 48 to 72 hours of the reaction where the analyses are straightforward.

The possible explanations for the apparent difficulty in inactivating the last traces of viable virus with formaldehyde in a suspension are myriad and, since quantitative studies in this area are difficult to design, much speculation exists. This speculative 3rd stage, C in Figure 6, is represented by the lag in inactivation of the very small proportion of "resistant" or "masked" virus particles. In our experience prolonged incubation with formaldehyde is required to produce completely inactive virus (up to 21 days in some cases). It might be postulated that, whatever the protective mechanism is that produces the "tailing" effect in the pattern, the last stages of formaldehyde inactivation procedure are completed by thermal effects rather than by the formaldehyde. In other words, formaldehyde inactivation alone as utilized is in reality a combination procedure. Evidence from heat inactivation studies indicates that variation in the virus population in susceptibility to elevated temperature is quite marked (45). Clumping or aggregation of virus particles or protection of virus by cellular debris is almost surely a contributing factor in the "tailing" of the formaldehyde "rate curve". Aggregation may be directly induced by the formalin treatment (39, 42), or some few virus particles, and the protein cell debris may be tanned or fixed by formalin, thereby rendering the virus incapable of infecting the cell until "unmasked" by enzymatic action in tissue. That is, a small proportion of virus may not actually be inactivated in a true sense by formaldehyde. However, lack of monodispersity is not entirely responsible for the "tailing" effect and the importance of resistant genetic variants, reactivation by reversal or recombination, or other unknown factors remain to be assessed.

The unique cytopathogenic delay effects observed with formaldehyde-treated virus, and the fact that formaldehyde inactivation

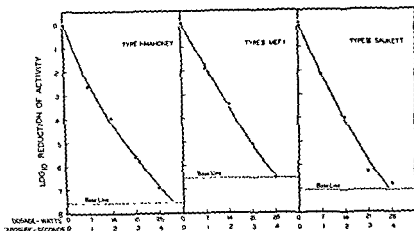


Fig. 8. Inactivation rate curves of three types of poliomyelitis virus using successive 7-watt increments of incident ultraviolet energy J. Immunol (17)

the reduction in film thickness. Another means whereby the inactivation rate curve could be made linear was by dilution of the tissue culture medium 199 with saline solutions. Neither the reduction of the rate of flow nor the dilution of the medium was of practical importance because both procedures resulted in reduced antigenic potential of the preparations (17)

3. Rate of Inactivation and Ultraviolet Absorption of Concentrated Virus in Buffered Saline

Concentrated poliovirus in buffered saline solution was ultraviolet-treated using the same 7-watt increments and revealed the same nonexponential relation seen in Figure 7. Here, however, the nonlinear relation was due to some degree to the absorption of ultraviolet by the virus itself (17) Comparison of the ultraviolet absorption spectra of irradiated to unirradiated virus revealed that the irradiation results in a shift of the characteristic nucleic acid absorption maximum at 260 mμ toward the shorter wavelengths. At the same time there is an increase in the absorption from wavelength 255 to 220 mμ and in the region 330 to 280 mμ. Thus, in highly concentrated and partially purified poliovirus suspensions, the development of

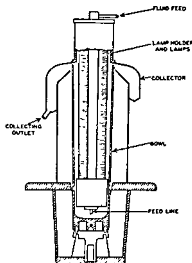


Fig 7. Diagrammatic vertical section of centrifugal filmer, "centri-Filmer", for the exposure of fluids to ultraviolet irradiation in the form of thin-flowing films J. Immunol (17)

thickness of the fluid film is proportional to the rate of liquid flow; at 100 ml./minute the average film thickness is approximately 15 microns; at 600 ml./minute, approximately 75 microns.

2. *Rate of Inactivation of Poliovirus in Medium 199*

Successive 7-watt increments of incident ultraviolet energy were applied to a series of filtered virus strain pools, irradiated at 600 ml. per minute, and the resulting inactivation rate curves are shown in Figure 8. Log reduction of activity (initial titer minus irradiated titer) is plotted against the time of exposure and also the dosage (17) of ultraviolet. None of the curves follows a constant exponential course; the initial increment produces an average log loss in titer of 2.25, the successive ones decreasing steadily until the 4th gives an average of only 1 log. Using the same sequence of 7-watt increments of incident ultraviolet energy and sampling, aliquot portions of strain pools of filtered tissue culture virus were irradiated at 2 slower rates of flow. At 300 and 150 ml. per minute, the rate of inactivation was much more rapid and appeared to be exponential, probably due to

careful consideration and evaluation of the effects of the absorption characteristics of the medium.

Experience gained in these studies has shown that if the virus titer of current poliovirus tissue culture fluids (medium 199) is reduced from 4 to 7 logs by means of ultraviolet irradiation at 15 to 25 watts of incident ultraviolet energy at a flow rate of 600 ml. per minute, this irradiated material is highly sensitive to other inactivation treatments. For instance, the virus may then be completely inactivated (negative safety test) by means of 10 to 16 days of mild heat treatment at 37° to 40°C. Such material retains a high degree of antigenicity (52, 53). In the following section it will be shown that a combination of formalin and ultraviolet may also be used to produce highly antigenic inactive virus rapidly and consistently. It should be pointed out that in all probability the mechanism of inactivation by ultraviolet involves the alteration of some degree of organization within the virus particle or "cell" (17) and undoubtedly is quite different from the mechanics of formaldehyde inactivation (7); the latter can be considered as a chemical type of inactivation (denaturation by increased bonding), while the former is predominantly a physical reaction (rupture and rearrangement). Both the formaldehyde and ultraviolet inactivation effects are depicted in Figure 2.

Exposure of the virus suspensions to the irradiation in the form of a thin film makes it possible to maintain an essentially uniform density throughout the film. Practically, it may be said all virus particles receive essentially the same exposure. Reflection from the bowl wall is a major factor in the over-all net exposure (17).

Earlier studies of the effect of ultraviolet irradiation upon viruses were carried out in quartz cells or open dishes, or in relatively thick films, and required prolonged exposures in terms of time: 15 to 40 minutes were required to produce 6 logs of loss in titer (17, 50, 51). Such irradiation techniques entail drastic over-irradiation of the bulk of the particles in suspension causing great loss of antigenicity as well as activity. With the present refinements and controls in the precision "Centri-Filmer", it is possible to introduce into a given volume of suspension in a matter of less than a second, the same quantity of ultraviolet energy which formerly required many minutes of exposure. This thin-film principal of irradiation has been used in these laboratories for a number of years for the preparation of vaccines with marked success. Such vaccines are relatively more highly antigenic and more stable than those inactivated with chemicals.

increased ultraviolet absorption in effect progressively "masks" the residual active virus (49).

4. Integration of Ultraviolet Irradiation Studies

That ultraviolet will produce inactivation of poliovirus is generally known (50), but data relating the level of the incident intensity applied to the irradiation effects upon the virus itself are meager. It was learned quite early in these studies that it was not practical to produce a complete kill of poliovirus with ultraviolet alone because the antigenic potency was seriously affected. Ultraviolet-treated virus was found to show spectroscopic evidence of internal rearrangement of the nucleic acid or nucleoprotein moiety of the virus and this, under controlled conditions, could be associated with loss of the infectivity while the antigenic properties were retained.

No attempt was made to treat the inactivation rate data (Figure 8) mathematically or to calculate the absolute quantity of energy involved in the virus inactivation because there has been no means of quantitatively estimating the relative amounts of ultraviolet energy absorbed by the virus and by the culture medium. Further, the film thickness in the filmer and the absolute exposure time are dependent upon a number of factors: viscosity, temperature, surface tension and frictional resistance to flow, and calculations of the allocation of the quantity of ultraviolet beyond the total incident energy level delivered at the film surface, as indicated in the detailed report (17), has little meaning beyond the theoretical.

Examination of the rate curves (Figure 8) will reveal that, within the limits of the error of the titrations, uniformity of effect is obtainable from lot to lot of virus. As has been suggested by FOGH (51), the departure from linearity observed might be due to the presence of virus particles especially resistant to the irradiation. However, factors other than the primary photochemical inactivation reaction are known to be operative and to affect the curves of the inactivation rate as measured by virus titrations (50). The relative over-all absorption of ultraviolet by the medium (containing amino acids) was far greater than that of the virus itself, the maximum occuring in the 260 mμ wavelength region. This observation served to emphasize the advantages of the thin film principle for the effective irradiation of infectious materials, and especially, it indicated the need for a

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Combination Inactivation Method for the Preparation of Poliovirus Vaccine

With the availability of more detailed information concerning the nature and dynamics of formaldehyde and ultraviolet inactivation of poliovirus and the progressive integration of this information with the difficulties encountered with the formalin inactivation process on a production scale, it appeared to us that a combination of methods might offer many advantages. Combination of 2 methods, each differing in mode of action and each alone capable of reducing the infectivity of poliovirus fluids by a factor of one million (10^{-6}) or more, should yield a safe product without overtreatment with either agent. It, therefore, seemed unlikely that any given infective particle in several million resistant to 1 method of inactivation would also be refractory to both to the same degree.

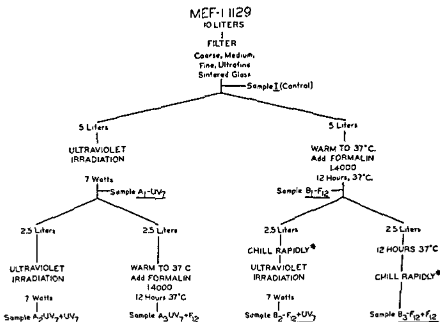


Fig. 9. Flow sheet detailing the plan of experiments in which 7-watt increments of ultraviolet irradiation were combined with 12-hour increments of formaldehyde inactivation in order to illustrate the fact that accumulative effects are obtained with the combined inactivation procedures. J. Immunol. (18)

In an extensive series of preliminary experiments (18), it was shown that a relatively short incubation period of 3 days at 37°C. with formaldehyde, either before or following ultraviolet irradiation (20 watts), would produce completely nonviable virus suspensions, mono- or trivalent, with excellent antigenic potential. All potency and safety tests were carried out according to the standards (14). An experiment representative of the type used to establish the combination inactivation procedure is described briefly below. This type of experiment establishes the interaction and alteration by the ultraviolet of the delayed initiation of infection phenomenon noted with formalin-treated virus, and illustrates the rationale for combining a chemical (formaldehyde) and a physical (ultraviolet) method of inactivation

1. *Accumulative Inactivation Effect of Formaldehyde and Ultraviolet*

When 7-watt increments of incident ultraviolet energy (17, 18) were combined with 12-hour increments of formalin (1:4000) inactivation at 37°C., it was possible to show that the effect appeared to be more than additive and possibly synergistic. The flow sheet (Figure 9) details the plan of the experiment. The samples at the respective levels of treatment were titrated in triplicate and showed an average spread of less than 0.3 log. The averages are plotted graphically in Figure 10. The combining of two 12-hour formalin increments (I-B_1-B_3), as expected, did not give twice the result of the first increment. TIMM ET AL. (7) have demonstrated that during the first few hours of formaldehyde inactivation the rate of titer loss is rapid, followed by a decreasing rate. The two 7-watt increments of ultraviolet are additive (A_1-A_2) again, as expected (17), for this virus concentration and part of the rate curve. The use of 7 watts of ultraviolet followed by 12 hours of formalin I-A_1-A_3 gives essentially additive results and about the same lowering of titer as two increments of either ultraviolet or formalin treatment. The greatest lowering of titer is seen in the combination of 12 hours of formalin followed by 7 watts of ultraviolet, I-B_1-B_2; the reduction in titer is a log greater than any of the other combinations. This result has proved to be quite consistent and may well be related to the known catalytic effect of ultraviolet light on many chemical reactions (34, 36). In other words, molecular disruption in the nucleo-protein portion of the virus is indicated by the spectrophotometric studies and, con-

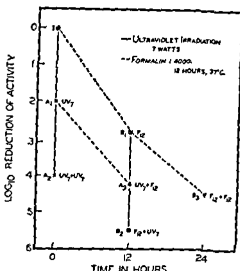


Fig. 10. Graphic recording of the log losses of activity obtained following the use of ultraviolet irradiation and formaldehyde increments detailed in Figure 9. Note that the combination of 12 hours of formaldehyde inactivation with seven watts of ultraviolet results in one log greater loss of titer than any of the other combinations. J. Immunol. (18)

jecturally, it may be said that the cyclic bonding of the formaldehyde reaction is quite probably potentiated in the protein moiety of the virus by the ultraviolet so that the normal course of the formaldehyde inactivation is enhanced. (Note Figure 2 where the 2 inactivation reaction concepts are compared.)

As pointed out earlier, formalin-treated virus in tissue culture fluid where the virus titer has been reduced several logs shows a delay in the initiation of the cytopathogenic effect when inoculated into susceptible monkey kidney tissue cultures. This finding is illustrated in Table II where the 3- and 7-day plaque titers of the control virus suspension I and the 4 final samples are tabulated. It will be observed that in the untreated control material, diluted approximately 4 logs, virtually all the plaques potentially available were present on the 3rd day of observation; very few more had grown out by the 7th day and a final 7/3 day ratio of 1.03 was obtained. The A_2 sample derived from the two 7-watt increments of ultraviolet irradiation was quite similar, showing no significant delay in the appearance of the total number of plaques. In sharp contrast is the result obtained with the B_3 sample which had received the two 12-hour increments

Table II. Plaques are counted on the third and seventh days and ratios obtained. The rate of the development of the plaques (cytopathogenicity) is influenced by the type of inactivation treatment. The delayed cytopathogenicity is characteristic to the formaldehyde J. Immunol. (18)

Accumulative inactivation – Formalin ultraviolet – Formalin effect on – Rate of plaque development

Sample	Dil n Tested	Titer per Ml.	Total Pfu - 4 bottles - 2 Ml.		Ratio 7 day / 3 day
			3rd day	7th day	
I Control	$10^{-5\,0}$	$10^{-6\,9}$	204	210	1 03
A_2 UV - UV	$10^{-1\,0}$	$10^{-2\,9}$	135	154	1.14
A_3 UV - F	$10^{-1\,0}$	$10^{-2\,7}$	41	106	2.58
B_2 F - UV	10^{-0}	$10^{-1\,4}$	9	55	6.11
B_3 F - F	$10^{-0\,5}$	$10^{-2\,4}$	15	168	11.20

of treatment with formaldehyde at 37°C. Here there is a most significant delay in the rate of plaque development. Only 15 plaques of a total of 168 eventually obtained on the 7th day were observed on the 3rd day. The ratio was 11.2 as compared with the 1.03 obtained for the control material. The ratios for the A_3 and B_2 samples where combinations of formalin and ultraviolet were used for the partial inactivation were intermediate. It is of particular interest that the ratio obtained, when the sequence was ultraviolet followed by formalin, was lower than that observed when the formalin was followed by the ultraviolet treatment. This phenomenon of delayed cytopathogenic response has been discussed in more detail above and, it should be recalled, is unique to formaldehyde treatment (7, 18, 19, 20).

2. Discussion of Production Experience with the Formaldehyde-Ultraviolet Method

In meeting desired vaccine standards (that is, the production of vaccines with consistent and acceptable safety and a high degree of antigenic potency), 2 years of costly trial and error had shown that the formalin process alone required repeated filtration and prolonged incubation, both potentially deleterious to antigenicity. In an interim

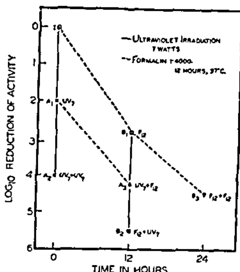


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days and observed at 3, 7, 10, 14, 17 and 21 days; positive tubes to be confirmed or ruled nonspecific. Blind passages were to be made from the original tubes at the end of 7 days and these passage tubes were to be subcultured (2nd blind passage) after 7 days of incubation. All tubes were discarded at 21 days. This test was required (February 1, 1954) for each strain component before pooling to form the trivalent vaccines for use in the field trial. An intracerebral test in monkeys was done on these final vaccines.

It was our belief that the tube culture procedure, as prescribed, did not test a sufficient quantity of vaccine to assure safety. The use of more tubes was impractical because of the large number of cultures involved so the bottle test was developed. Starting on February 1, 1954, in addition to the tube test above, two 16-ounce bottles (MKC) were inoculated with 25 ml of dialyzed strain component, plus 75 ml. of medium 199, and observed for 28 days and subcultured at 7, 14, and 21 days. The subcultures, 0 5 ml /tube, 5 tubes per bottle were observed for 7 days. *Two control bottles were carried in parallel.* On March 22, 1954 this test was extended by the addition of one more bottle and the bottle size increased to 32 ounces with 50 ml. of vaccine tested. On May 20, 1954 (first working draft of Minimum Requirements, U S. Public Health Service) the bottle test was made official and increased to 5 or more containers (effective volume 0.1%) and applied to both trivalent vaccines and strain pools. In this official version, however, the bottles were to be held only 14 days.

The specific experimental studies discussed above, particularly the delayed cytopathogenic phenomenon, provided the rationale and led to the attempts to improve the sensitivity of the test by: 1) testing much larger volumes of vaccine in larger containers – 32-ounce prescription bottles, and 2) by holding the test for as long a period as practicable. Since July 14, 1954, all routine poliovirus vaccine production in this laboratory has been tested as follows: at least 5 or more original culture bottles are inoculated with 50 ml. of sample and 50 ml. of medium 199, held for a total of 28 days with weekly subcultures. This, with the 28-day subculture, makes the total duration of the test 35 days. The trivalent vaccines are tissue culture safety tested in duplicate on at least 2 different cell lots. Details are contained in the earlier report (19).

This combination of formaldehyde inactivation with ultraviolet irradiation resulted in a safe and effective vaccine. To obtain comparable negative safety tests with a reasonable degree of consistency on material inactivated by formalin alone requires, according to our experience, at least 12 to 21 days of incubation at 37°C. Even with this prolonged treatment occasional active strain pools were encountered. Further, material on which the initial strain pool test was negative often showed up positive on later tests in the trivalent stage.

The incorporation of repeated filtration and clarification procedures (4, 13) is quite effective in reducing the persistent cytopathogenic virus in formalin-treated materials (contained in aggregates), but may have an adverse effect on the over-all potency. This appears to be true especially of the Saukett strain, which is not only more sensitive to both ultraviolet and formalin, but appears relatively more susceptible to removal from solution when inactivated or partially inactivated formalinized or untreated virus solutions are filtered (7). The other 2 strains (Mahoney and MEF-1) are affected likewise, but to a lesser degree.

The manufacturing experience under the revised requirements (14) indicates that a safe vaccine can be produced with some sacrifice of potency by using formaldehyde inactivation incorporated with *repeated filtration*, *extended formaldehyde treatment*, and *increased safety testing*. Statistical analyses of the results point out that, even though safety and consistency have been established, the safety tests (both tissue culture and cortisone monkey) must be the final critical line of appraisal (15).

Poliovirus Vaccine Safety Testing

Even before the development of the reliable large-scale combination inactivation method for poliovirus, it was found necessary to develop a consistent method of testing for the nonviability of the poliovirus in inactivated material. At first (September 18, 1953), tissue culture safety tests used for the preliminary studies were carried out in monkey kidney cell (MKC) tube cultures (0.5 ml. vaccine samples in at least 10 tubes held for 10 days and observed daily). No subcultures were made, but any cell degeneration observed was confirmed as positive or negative for virus. On January 13, 1954 this test was extented to consist of 40 tubes (20 ml. tested) held for 21

Table III. Early vaccine lots produced by the use of formaldehyde inactivation alone are compared in parallel safety tests with and without serum in the medium. No specific virus inhibition in these tests can be attributed to the presence of the serum. Note that in vaccines 1, 3 and 5 the majority of positives occur at 21 days or later. J. Immunol. (19).

Summary of positive tissue culture safety tests on trivalent pools

Vaccine No.	No. Bottle on Test	Medium 199 Containing	No. Bottles Positive at (days)				Type Isolated
			7	14	21	28	
1	17	0 5% Calf serum	0	0	3	0	1
	14	No serum	0	1	1	0	2, 1
2	14	0.5% Calf serum	0	4	0	0	1, 2, 3
	17	No serum	0	8	1	0	1
3	17	No serum	0	2	3	2	2
4	29	0.5% serum	1	1	0	0	2
5	52	0 5% serum	0	0	4	0	2, 3

sensitivity are considered. Note the multiplicity of strains found and the length of time required to demonstrate them. Most of the positive cultures were found at the time of the 14- and 21-day subcultures, but in vaccine 5, positives were not observed before the 21st day subcultures and in vaccine 3, the majority of the positives occurred in the 21- and 28-day subcultures. The multiplicity of strains appearing in these vaccines is especially noteworthy. In 4 cultures of the 31 found positive among 160 on test, more than one type of virus was recovered. This finding is not improbable on the basis of chance alone However, when one considers that the original strain pool tests were negative, it invites speculation on the possibility of intertype reactivation. Possibly certain conditions could permit reactivation of "inactivated" particles or the multiple strains observed could conceivably arise from the superinfection of some cells by more than one partially inactivated particle. It is known that the exposure of susceptible cells to two or more inactivated viruses sometimes results in live progeny (56, 57). It is difficult under comparable conditions to design experiments to test such a possibility because of the very low incidence of such occurrences. The phenomenon of delayed plaque appearance described above due to prior treatment of the virus with formalin is also in the picture and it would seem advisable to exercise the utmost precaution in the design of the safety test for poliovirus (58).

1. Safety Tests on Materials Inactivated with Formaldehyde Only

Of 469 strain pools inactivated with formaldehyde alone (October 1954 – October 1955) for periods varying from 7 to 17 days, the strain pool type was recovered in all cases where positives occurred. Considered as a group, the total percentage of tests with positive 21- or 28-day subcultures was: Type I, 15.5%; Type II, 17.2%; and Type III, 11.5%. Some of these also showed earlier positives in the same series of test cultures, but if those tests showing only late positives (21st day or later) were considered, the percentages were lower but still very significant: Type I, 9 tests or 6.1%; Type II, 15 tests or 8.6%; and Type III, 5 tests or 3.4%. In other words, a total of 29 of the 469 strain pools would have been considered negative for virus and carried into trivalent vaccines had the 21- and 28-day subcultures not been run (19). This late demonstration of living virus in formalin-treated suspensions both mono- and trivalent is coupled with the fact that the more prolonged the formaldehyde treatment the more delayed the appearance of the cytopathogenic effect becomes (Figure 2 of Reference 20). This is confirmed also when the data from these tests are expressed as the number of effective bottles positive at the time of the weekly subcultures. In this case, all tests were considered as terminated with the appearance of the first positive bottle (Table 5A of Reference 19). The percentage of positive bottles first observed in each test decreased from 8.27 on the 7th day to 5.28 to 2.32 to 1.08 for the 2nd to 4th weeks, respectively. In 30 of 2777 cases, the 1st positives were detected in the 28th day subcultures. Thus with the prolonged formaldehyde treatment up to a total of 21 days, delay in the appearance of positives was observed to at least 28 days. The corresponding trivalent vaccines also showed a similar delayed cytopathogenic effect with the highest instance of 1st positive bottles (2.94%) appearing in the 21st day subcultures. In this case only 0.44% of the positives was evident by the 7th day. Each of these vaccines was prepared from strain components that had appeared inactive on the prevous individual strain pool tests. (*Also 28 days* plus subculture.)

The combined results of the total tissue culture safety testing of 5 of the early trivalent vaccines which were found to be positive are summarized in Table III. No specific virus inhibition in these tests can be attributed to the presence or absence of serum (19) when the influence of sampling techniques and probable variations in cell

cedure of the cortisone treatment and intraspinal inoculation has further increased the sensitivity of the animal test (10, 11, 12). However, no positive monkeys have been encountered on our production lots since the introduction of the prolonged tissue culture safety test, using adequate test volumes, for all strain component lots prior to their inclusion in trivalent vaccines.

Filtration in the Inactivation Procedures

The additional filtration requirements introduced into the process to achieve "consistency" (9, 13, 41, 60) were justified as being necessary to establish the "theoretical projection". It was also stated that differences in filtration practices and technical details of the application of the original process to manufacturing were responsible for certain inconsistencies in the commercial vaccines. There is little question but that the filtration can contribute to the obtaining of consistently negative safety tests. However, its introduction as an expedient measure did not acknowledge the probable major source of the difficulty.

Although repeated filtration (13) and the combined inactivation process (18) were introduced simultaneously, the results indicate to us that the latter was the more important change. On a limited scale in the laboratory (18), it was possible to inactivate unfiltered poliovirus tissue culture fluid with the combined treatment. Aliquots of the same material treated in all other respects by the standard formalin procedure (9, 14) were uniformly positive. In addition, extensive repeated filtrations do not eliminate the delayed cytopathogenic effect observed for formalin-treated poliovirus and other viruses (20). This would indicate clearly that the removal of occluded virus ("solid phase") by filtration resolves only part of the difficulty. Prolonged incubation of tissue culture virus suspensions with formaldehyde alone produces a progressive increase in the size of particulates (39) which can be shown photometrically (42). The importance of this phenomenon and the problem of aggregates in general is shown by additional unpublished experiments from this laboratory such as the following: A sample (600 ml.) of a poliovirus preparation that still showed an occasional positive bottle on safety test after 17 days of formaldehyde treatment was angle centrifuged at 6000 X gravity for 2 hours. The upper portion of the fluid from each tube was removed

2. *Safety Tests on Materials Inactivated with the Formaldehyde-Ultraviolet Combination Method.*

In these laboratories, after October 1955, all poliovirus for the preparation of vaccines was inactivated by the combination process (18). In July 1956, the quadruple ultra-fine sintered glass filtration was replaced by double Seitz filtration. These changes, both in the inactivation process and degree of filtration, resulted in a dramatic reduction in the number of inactivation failures. Furthermore, while the over-all number of positives is small, there is clearly less of a tendency for the appearance of late positives. This was anticipated since it had been shown that the combined treatment resulted in a significant decrease in plaque delay time associated with an equivalent degree of inactivation achieved by formaldehyde alone (18, 20). This is clearly illustrated in the data documented in Table 5 of Beardmore et al. (19). Of 7727 bottles tested beyond 14 days, only 1 positive was observed. In contrast, the earlier materials treated with formaldehyde alone had 132 positives among 4085 bottles in test beyond 14 days.

These data indicate the importance of utilizing an extended observation period beyond the 14-day subculture (14) for the tissue safety test.

3. *Safety Testing in Monkeys.*

The inadequacies of the original monkey safety test have been reviewed (59). However, it is striking that many of the positive vaccines were detected by this procedure (13). With nonformalinized poliovirus it can be demonstrated readily that tissue culture methods are a thousand- to a millionfold more sensitive. Unpublished experiments, done in this laboratory, indicate that this difference in sensitivity (monkey vs. tissue culture) diminishes progressively during the process of formaldehyde inactivation. Type I poliovirus was treated to "base line" levels with formaldehyde and inoculated into monkeys and tissue cultures simultaneously. The residual activity can be detected equally well in the two systems at this stage of inactivation. We cannot resolve at this time whether the demonstrable change in relative sensitivity is due to particulated material or the delayed cytopathogenic phenomenon. It is interesting to speculate that *in vivo* conditions are more conducive to the unmasking of occluded or chemically damaged virus. The introduction into the original pro-

basic working tools, but it has been our experience that different serum preparations give varying estimates of potency on the same series of antigen preparations. Thus, at present, uncontrollable variables negate comparison of results from test to test if the serum lot is changed. The tests that depend on measuring the level of antibody response in a limited group of animals (including the standard monkey potency test) are inadequate for day-to-day quantitative use because they reflect too greatly the varying response potential of the test animals at different times and under different conditions of nutrition, health, previous antigenic experience, etc. The most promising have been those procedures employing graded dose, particularly the guinea pig assay suggested by GARD (69). This test is of particular value for experimental work since guinea pigs do not respond differentially to active or inactive poliovirus. Unfortunately, the relatively high cost of guinea pigs and the difficulty of maintaining large numbers under crowded conditions makes use of adequate number per dose impractical for process control.

In July, 1955, a series of experiments were undertaken to compare the responsiveness of a number of readily available laboratory animals to active and inactive poliovirus. In the course of this work, it was found that 7- to 21-day old chicks responded well and uniformly. Since then, baby chicks have been used extensively in this laboratory for the evaluation of experimental procedures on poliovirus antigen and have proven satisfactory in every regard (21) They are readily available and inexpensive, large numbers can be confined in a small space, and most standard breeds are highly inbred and genetically uniform. There is a difference in responsiveness between breeds and the so-called "egg-breeds" (e. g., White Leghorns) give lower levels of antibody than the "meat-breeds" (e. g., barred Rocks) (70). Either a single inoculation or multiple inoculation schedule may be used and optimal bleeding time is about 5 days following the last dose, or even earlier in the case of a single dose. We have found it most convenient to sacrifice the birds and collect blood directly from the slit jugular vein.

Recently the American Drug Manufacturers Association and the Division of Biologics Standards, of the National Institutes of Health, have been engaged in a large-scale cooperative evaluation of the use of baby chicks on an antigen dilution type potency test for poliovirus vaccine. This program seems to be giving quite promising data and may well result in a revision of the current potency requirements.

carefully and pooled. The lower portions were also pooled and the two 300 ml. samples safety tested in parallel on 6 MKC bottle cultures each. The fluid from the upper portion of the tubes was completely negative, while the lower portion showed 4 positives after a prolonged incubation period. This cycle sequence in the centrifuge would sediment particulates the size and density of rickettsia and larger, but not monodispersed poliovirus. Parenthetically, particles of this order of size (rickettsia) could contain possibly 1000 virus particles. Particulates of this type are illustrated in the electron micrograph shown in Figure 4. It is of interest to note that these agglomerates measure approximately 250–350 mμ and were obtained by centrifugation of *polio vaccine immediately following ultra-fine sintered glass filtration.* These results were instrumental in the incorporation of the centrifugal clarification into the combined process (18).

Filtration during the course of inactivation, as is currently required (14), has certainly contributed to the safety of the commercial vaccines. However, the Saukett strain, in particular, is more readily removed by filtration and is also more susceptible to both formaldehyde and ultraviolet inactivation (7, 18). The current question of the possible inadequate potency of recent commercial vaccines may well be a result of the increased filtration when combined with prolonged formalin treatment. Thus, the "adequate margin of safety" (9, 41) claimed was achieved eventually, but at the expense of the potential potency of the vaccines. That is, increasing the filtration requirement *without consideration of all aspects of the mechanisms involved in* the inactivation procedures may have resulted in a safer but less potent vaccine.

Potency Testing of Poliovirus Vaccine

In the development of the combined formalin-ultraviolet inactivation procedure, the lack of a truly quantitative and consistent method *of estimating potency was a serious handicap.* Many tests have been proposed which are based on graded antibody response, or on the 50% response level to graded vaccine doses in animals (53, 61–69), or on the *in vitro* estimation of antigen content by either complement-fixation (70) or antibody-combining capacity (71).

These methods have all been investigated and were found inadequate for our purpose. The *in vitro* procedures are promising as

Table IV. Effect of thimerosal on infectivity of poliovirus type II (MEF-I strain)

	T. C. (MKC) Titers After Holding At 36°C. For:				
	0 Days	2 Days	4 Days	6 Days	8 Days
1 Virus Alone	5 5	4 6	3 8	3 7	3 2
2. Plus 1 5000 Thimerosal	4 6	Trace	Neg	Neg	Neg
3 Plus 1.10000 Thimerosal	5 2	3 6	Neg	Neg	Neg
4 Plus 1 20000 Thimerosal	4 9	4 2	Trace	Neg	Neg

rather to mercurial degradation products, the formation of which was catalyzed by certain metallic ions (e. g., copper). If highly purified thimerosal was used and a chelating agent also added, then the degradation was greatly retarded and a satisfactory shelf-life for the preserved poliovirus vaccine resulted Several manufacturers, therefore, continued to use thimerosal in their product, but also added dihydro-disodium ethylene-diaminetetra-acetic acid* to chelate catalytic heavy metals. If this procedure is followed, it is imperative that only freshly prepared thimerosal be used, otherwise degradation products may already be present.

As soon as the cause of the vaccine instability was found, we undertook an intensive investigation in search of alternate preservatives The most suitable proved to be purified and recrystallized benzethonium chloride** (74). This compound, when used in concentrations ranging from 1:20,000 to 1:100,000 is an efficient bacteriostatic and fungistatic agent and does not decrease the stability of the poliovirus antigen In fact, we have stored active poliovirus, Type I, for over 18 months at refrigerator temperature in the presence of 1:10,000 benzethonium chloride with no loss of infective titer.

Summary and Conclusions

1. The cultivation of poliovirus on monolayers of trypsinized monkey kidney cells when compared with suspended cell cultures yields harvest fluids with greater quantities of virus and contains 1/5 to 1/10 the total quantity of extraneous monkey cell protein.

* Trade-mark name· Versene; Versenes Inc., Framingham, Mass.
** Trade-mark name· Phemerol, Parke, Davis and Co , Detroit, Mich.

Preservatives for Poliovirus Vaccine

The first requirements for poliovirus vaccine specified the use of 0.01% thimerosal* as a preservative since multiple dose containers were to be used for the field trial vaccines. Following the preparation and use of these lots in the trials, it was found in this laboratory that even after short periods of storage a serious loss of potency had occurred. This effect was most marked with the Type I component, particularly if at any time the product had been held for a period above refrigeration temperature. The deleterious effect was more prominent with lots prepared on trypsin cell monolayers because of the lower extraneous protein content of these materials. On stability studies with certain of the field trial lots, we found that a detectable drop in potency for Type I occurred after 2 weeks of storage at 4–8°C. and almost all of the Types I and III antigen were destroyed after a day or two at room temperature. As soon as these findings had been confirmed (41) the specifications were changed making the use of a preservative optional. However, a large amount of vaccine already prepared for use in the contemplated 1955 studies was lost.

It was impossible, at this late date, to assess the effect of the inclusion of thimerosal in the vaccine on the results of the 1954 Field Trials. The different lots of vaccine were used after varying periods of storage and the manner in which they were handled in the field also was extremely variable. Certainly, in all cases, the results would have been more uniform if the potency had not been degraded to an undetermined degree by varying storage time and handling. The mercurial preservatives are potent inactivators of poliovirus (72) and earlier it had been considered that possibly the thimerosal might be added at the same time as the formaldehyde in order to assist in the inactivation process. Table IV illustrates the effect of thimerosal on the infectious titer of Type II poliovirus. With the 1:10,000 concentration (employed as preservative in early vaccines) no infectivity was demonstrable after 4 days. Similar results were obtained with Types I and III. Thus, one might speculate that the use of thimerosal as a preservative in the early vaccines may have contributed to the inactivation as well as the loss in potency.

It was later demonstrated (73) that the potency loss observed in the vaccines was not due to the effect of the thimerosal directly, but

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* Trade-mark name: Merthiolate; Eli Lilly and Co., Indianapolis, Ind.

Association (December, 1957) that: – "Three years of increasingly widespread use of... (Salk) polio vaccine have coincided with unprecedented reduction in reported cases of poliomyelitis (in the United States). A particularly low incidence and low paralytic rate are the outstanding features of the 1957 season."

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2. Departure from a linear relation obviates the use of inactivation rate titrations as an indication of the time required for total inactivation of the virus in the production of vaccines.

3. Formaldehyde-treated poliovirus shows a unique delay in the initiation of cellular infection when it is inoculated into susceptible tissue cultures. This effect influences the results in both titrations and bottle cultures used in safety testing.

4. *Careful analyses of the inactivation rate curves revealed that there* are at least two, possibly more, distinct stages in the formaldehyde inactivation of poliovirus. Quite probably a combination of chemical and thermal reactions is involved. Theoretical and practical implications are discussed.

5. Controlled ultraviolet irradiation of poliovirus fluids results in the loss of the major portion of the virus activity and produces a marked increase in the sensitivity of the virus to chemical inactivation.

6. Ultraviolet irradiation, when used in combination with formaldehyde for inactivation, reduces the characteristic delay in cytopathogenic effect due to formaldehyde treatment.

7. A consistent and reliable combination inactivation procedure (formaldehyde-ultraviolet) for the production of vaccines was evolved within the limits of the regulations. Compared in potency and antigenicity with previous products (formaldehyde only), the product was shown to have at least equal, in most cases greater, stability and antigenic potency in both animals and humans. Theoretical and practical aspects are discussed.

8. *Development of, and the practical utilization and interpretation* of, a reliable tissue culture safety test was found to be dependent upon the integration of fundamental facts regarding the mechanics of the inactivation procedures (for example, the unique delay in cytopathogenic effect produced by formaldehyde-treated virus).

9. A 28-day tissue culture safety test in bottles, subcultured at weekly intervals (total test period 35 days), was developed and, when *used with the combination inactivation procedure, no positive safety* tests have been detected in more than 200 strain pools produced (over 6200 tested culture bottles).

10. The use and effectiveness of preservatives for poliomyelitis vaccines are discussed and evaluated.

11. In conclusion, the over-all effectiveness of poliovirus vaccination on a large scale appears to have been firmly established. CORIELL reported (75) to the 11th clinical session of the American Medical

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INFLUENZA, 1957 A VARIANT AND THE PANDEMIC

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"... *The one great feature of influenza which has not been manifested in the period of effective research is the major pandemic Until the natural history of such an episode has been elucidated, it will be impossible either to understand the whole influenza situation or to devise measures to minimize the effect of a subsequent pandemic* " (BURNET, 1950)

I. Introduction

The 1957 pandemic is the first since the development of virology as a science. Tools and concepts, which were unknown at the time of the 1918–1919 pandemic, have provided virologists and epidemiologists with unparalleled opportunities for study of a unique phenomenon. After almost 25 years of laboratory experience with etiologic agents of influenza and development of diagnostic and control methods, ideal conditions suddenly were presented. Concepts developed from studies of influenza and of its etiologic agents have been successfully applied to problems in a wide range of communicable diseases, but the crucial test in a pandemic situation had not yet been carried

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INFLUENZA, 1957
A VARIANT AND THE PANDEMIC

By KEITH E. JENSEN, FREDERICK L. DUNN, and ROSLYN Q. ROBINSON

"*.. The one great feature of influenza which has not been manifested in the period of effective research is the major pandemic Until the natural history of such an episode has been elucidated, it will be impossible either to understand the whole influenza situation or to devise measures to minimize the effect of a subsequent pandemic*" (BURNET, 1950)

I. Introduction

The 1957 pandemic is the first since the development of virology as a science. Tools and concepts, which were unknown at the time of the 1918–1919 pandemic, have provided virologists and epidemiologists with unparalleled opportunities for study of a unique phenomenon. After almost 25 years of laboratory experience with etiologic agents of influenza and development of diagnostic and control methods, ideal conditions suddenly were presented. Concepts developed from studies of influenza and of its etiologic agents have been successfully applied to problems in a wide range of communicable diseases, but the crucial test in a pandemic situation had not yet been carried

out. There was a sense of urgency in many minds, therefore, that full advantage be taken of all available techniques, knowledge, and facilities in prompt and rational action (BURNEY, 1957).

When it became apparent that pandemic influenza could be expected again in 1957 on the basis of laboratory and epidemiologic intelligence, personal memories of the 1918 disaster burned brightly and for some people there were moments of panic. The world will not soon forget that series of epidemics which gave meaning to the word "pandemic" as influenza swept through country after country. Almost everyone who is at least 50 years old today can remember the influenza which attacked half of the population of the world. Frequently they can recall a death in the family or in their circle of friends as the world death total mounted to more than 20 millions. Since that time, however, the disease usually has been so much milder that it has often been confused with common colds and intestinal upsets.

The A/Asian strains of influenza virus have produced a true pandemic since their first appearance in China in February, 1957. This pandemic has fulfilled the requirements set forth by FROST (1919): "The special characteristics of influenza pandemics are their wide and rapid extension, their high attack rates, and their great effects upon general mortality rates." While the mortality rates have been affected strikingly, they do not approach those of 1918–1919. Furthermore, there are significant differences in the age distribution of fatal cases for the two pandemics Epidemiologically, the 1957 pandemic resembled the pandemic of 1889–91 far more than that of 1918–19. Curiously enough, serological studies suggest that the virus responsible for the disease of 1889–91 may be rather closely related to the new strain antigenically (MULDER, 1956; JENSEN ET AL., 1957; DAVENPORT, 1957a; BOGER AND LIU, 1957)

At this time we cannot hope to present more than a partial recounting of the ensuing activities in the fields of virology and epidemiology which took place during the pandemic and are still continuing. Newspaper accounts and other communication media have kept many persons informed concerning the early history of the pandemic, but a more complete picture is now available and will be delineated in the initial sections of this report. The plan has been to present a review of the epidemiological aspects first and then to describe studies with the viral agents. Later sections are concerned with problems of vaccine production, standardization, and evaluation. Throughout this communication the reader should be advised

that we cannot attempt a comprehensive review; it is our intention only to present a progress report based on information which has become available to us.

II. International Spread of Asian Strain Influenza

The summary presented below was compiled from information received by the Influenza Surveillance Unit of the Communicable Disease Center, U. S. Public Health Service. Much of this information is preliminary and, in some cases, fragmentary. Sources of information included. Personal communication from many authorities; World Health Organization Weekly Epidemiological Records; Foreign Epidemiological Summaries of the U. S. Public Health Service; Morbidity and Mortality Reports, U. S. National Office of Vital Statistics; International Cooperation Administration Influenza Reports; dispatches from the major wire services.

The precise point of origin of the new strain of type A influenza will probably never be determined. It is reasonably certain, however, that the first outbreaks occurred in the hinterland of China (TANG AND LIANG, 1957). The disease was first reported in Kweichow province near Kweiyang at the end of February, and by the first week of March it had spread into Yunan province. By the middle of March the new strain was reported to be prevalent all over China. Asian strain viruses were isolated in Peking, Chang-Chia-Kow, Lo-Yang, and Changchun. An isolate forwarded by TANG to the Influenza Centre in London was identified as a typical Asian strain (PAYNE, 1957).

Early in April the disease appeared in Hong Kong in epidemic form, perhaps carried there by travellers from the mainland of China. Soon after the onset of the Hong Kong epidemic new epidemics were reported in Taiwan, Singapore (LIM ET AL., 1957), and Borneo. Although the virus was introduced at this time into Japan, an epidemic did not immediately take place in that country. Virus isolates obtained from these early epidemics and characterized in several laboratories (MEYER ET AL., 1957; JENSEN, 1957a; DAVENPORT, 1957; ISAACS, 1957; HALE, 1957) alerted the world for the possibility of a new pandemic.

During May many additional countries of eastern Asia reported epidemic influenza, in most cases confirmed by virus isolation. Malaya, the Philippines (TAYBACK AND REYES, 1957), Sarawak,

North Borneo, Australia, Okinawa, *Guam*, *Cambodia*, *India*, and Indonesia were affected during the month. In some cases it was possible to identify ships or aircraft, bearing passengers and crewmen with influenza, as the means responsible for the transmission of the disease to new countries. Opportunities for transmission, however, were *innumerable and far more remained unrecognized than were detected.* No amount of quarantining could have prevented the spread of the disease. During late May a number of cases of confirmed Asian strain influenza were reported among travellers from the Far East recently arrived in the United States. By the end of May two of the earliest epidemic areas, Hong Kong and Borneo, reported a marked subsidence of the disease.

In June at least 24 countries of eastern and western Asia, East Africa, and Europe were affected for the first time. A distinct westward pattern of spread was apparent in Asia as the disease moved from Burma and India through Pakistan into Iran, Iraq, Saudi Arabia, Bahrein, Yemen, and Aden (FAWDRY, 1957). At this time Moslem pilgrims bound for Mecca were known to have played a role in the transmission of the virus westward from West Pakistan. Late in the month the disease jumped the Red Sea to Eritrea and Mozambique on the east coast of Africa. A number of confirmed cases arrived in the Netherlands from Indonesia by ship and aircraft, and Romania and Czechoslovakia reported outbreaks during the month. Ceylon and Korea became involved, and the United States and Alaska reported frequent new introductions and localized outbreaks. By the end of June most of the countries first experiencing influenza in May had passed their epidemic peaks and reported declines. Japan reported an epidemic peak in mid-month, principally involving school children. The epidemics in Taiwan and Singapore were at an end early in June.

In July major spread occurred in the Middle East where Syria, Jordan, Lebanon, Kuwait (GUTHRIE ET AL., 1957), and Afghanistan were affected, and in eastern Africa where French Somaliland, Sudan, Egypt, and South Africa became involved. During the month declines from epidemic peaks occurred in Thailand, Laos, Vietnam, Burma, and Korea. Slow dissemination of the virus continued in the United

characteristically mild illness in camps, military installations, and institutions of various types. In Australia, which was first affected in

May, the vast size of the land and the scattered population perhaps contributed to the relatively slow buildup of the epidemic. Some areas were little affected even at the end of July. New Zealand reported the first outbreaks during this month and reports began to come to the World Health Organization from the widely scattered islands of the southern and western Pacific region.

Without any proven sources of introduction epidemic Asian strain influenza appeared dramatically in South America early in July. Bolivia and Ecuador were the first countries to report epidemics, but by the end of the month Chile, Colombia, Panama, and Mexico were also affected. The epidemics in Chile (HOUSER, 1957) and Bolivia were particularly severe and widespread, while the countries nearer the equator were apparently less heavily affected. Outbreaks in such diverse places as southern Italy, Greenland, Mauritius, and Newfoundland appeared before the month was over, demonstrating strikingly the importance of modern means of transportation in spreading the infection around the globe.

The month of August saw continuing epidemic decline in the Middle East and eastern Asia. Epidemic influenza was on the upswing, however, in the rest of the world. In South America, as the Chilean epidemic gained momentum, Argentina and Uruguay also became heavily involved. Later in the month British Guiana, Peru, southern Brazil, Trinidad, and, in Central America, Costa Rica and El Salvador reported outbreaks or epidemics. Puerto Rico noted the onset of an epidemic late in the month that was to sweep the island with great vigor during September. Europe and North America reported their first true epidemics. The introductions of influenza into southern Italy in July led to the first country-wide European epidemic in August and September. In the United States, the first major community epidemics appeared in several southeastern states in early August. Elsewhere in the world many new islands of Oceania were affected during August, and in Africa, Kenya and Tanganyika joined the other epidemic countries of the east coast. The interior of the continent apparently remained free of influenza as the disease jumped to the west coast (to French West Africa, Senegal, Nigeria, and the Ivory Coast) during the month.

September was characterized by massive invasion of Europe, North America, and northern South America. Only Liberia joined the list of epidemic countries in Africa. Except for Egypt, the north coast remained little affected while across the Mediterranean influenza

swept through Italy, Sicily, and flared up in Switzerland, Belgium, Greece, France, Poland, Bulgaria, and Portugal. England (BRADLEY, 1957) and the Netherlands began to experience an increase in the weekly number of cases after several months of sporadic occurrences. During September it was learned that outbreaks earlier in the summer in Yugoslavia and Romania had been confirmed as due to the new Asian strain. While the countries of the southern half of South America reported the ends of their epidemics, French Guiana, Surinam, northern Brazil, and Venezuela were in the midst of epidemics during September. Guatemala, Honduras, and a number of Caribbean islands were also heavily involved during the month. In the United States and Canada, with the opening of schools, influenza rapidly became widespread. Canada, like the United States, had noted increasing sporadic illness and a few local outbreaks during the summer (LOSSING, 1957).

By late September the Middle East and eastern Asia were virtually free of epidemic Asian strain influenza. In Australia decline was apparent after a prolonged epidemic which eventually affected all states. It is of interest that Israel, relatively isolated from contact with the rest of the Middle East, only noted the onset of an epidemic during this month when the other countries of the area were reporting declines or absence of influenza.

October was a peak epidemic month for Europe and North America. Virtually every country of Europe reported the onset or peak of an epidemic at some time during the month. The Scandinavian countries, as a group, were the last to be affected. The USSR apparently experienced a widespread epidemic during October also, although previously isolations from sporadic cases had been reported from various parts of the country. An A/Asian strain isolated near Moscow in May was made available for study in July at the Influenza Center for the Americas.

The epidemics or outbreaks in the Caribbean continued with some vigor but most South American countries were free of epidemic influenza by this time. Only Paraguay, geographically isolated, became affected for the first time during this month. In Africa, as in South America, the epidemic areas reported declines by late October. Ghana and the Belgian Congo were the only west coast countries with active disease at the end of the month, and most other African areas were epidemic-free, the north coast had still reportedly experienced little more than sporadic illnesses and outbreaks.

In Asia, only Japan reported epidemic Asian strain influenza during October. Japan, a northern Asiatic country, had experienced an epidemic of apparent sizeable proportions during the spring while most other areas of the northern hemisphere remained free of the disease. This first epidemic reportedly peaked in mid-June and had ended by mid-July. August was an influenza-free month but in September influenza again began to be reported and within a few weeks a sizeable excess mortality had built up. Reports indicate that hundreds of schools were affected. It is not yet clear whether this second wave of Asian strain influenza (the virus has been identified frequently from both epidemics) has affected primarily those individuals who were not attacked in May-June-July (that is, a split epidemic), or if this represents a true second wave with a high proportion of reinfections. Preliminary studies suggest that reinfections may be relatively rare (KOJIMA, 1957). No other country has reported such a second wave of influenza although certain communities in various countries (West Pakistan, Lebanon, Ecuador) have described second outbreaks. In none of these areas have the necessary virus confirmations been made, however, as they have in Japan. In late October even Antarctica was struck by febrile respiratory illness in epidemic form with the arrival of spring supply ships.

November was a month of epidemic decline in almost all parts of the world. North America and Europe were clearly less heavily involved than in October. No countries reported new epidemics or second epidemics, and it seemed probable at the end of the month that the pandemic was dying out. Every continent and almost every country and major island had experienced epidemic influenza by the middle of November. Only a few isolated islands and remote countries had not reported outbreaks. In Africa a large portion of central and north Africa had remained apparently unaffected. It is very likely, however, that lack of reporting rather than non-occurrence of influenza is responsible for most of the gaps in the records. A final analysis of the international spread of the new strain will not be possible until many months have passed after the end of the pandemic.

If any generalization can be drawn from this type of discussion and review, it is that season has been the most important determinant of the distribution of the new strain of influenza. During our spring and summer the pandemic was clearly restricted to the southern hemisphere where the winter season was in progress. The countries of Europe and North America, and even northern South America, were

not affected until the arrival of fall when suddenly and dramatically an upsurge of illness took place. How much this seasonal correlation is related to school attendance patterns is not clear at this time. In the United States the onset of the epidemic correlates well with the opening of the schools regardless of the climatic variations from state to state. Conditions of crowding have also contributed to the pattern of epidemics this year. Clearly, where crowding is prevalent, attack rates have generally been higher and spread has been more rapid. While crowding has been an important determinant of attack rate and rapidity of spread it has not, however, affected distribution of the disease in the final analysis, for virtually every corner of the world has been affected regardless of population density. These points are elaborated further in the next section which is devoted to influenza epidemiology.

III. Epidemiological Notes

Asian strain influenza affected virtually every major country in the world within six months after the disease spread from the mainland of China to Hong Kong. The virus was disseminated extensively around the world within three months, however conditions were not ripe at that time for epidemic influenza to occur in many of these countries. The spread of the virus through Europe and North America during the summer months set the stage for epidemic disease with the arrival of the fall season. The six-month period required for worldwide epidemic spread contrasts with the eleven months or more required for a roughly similar degree of spread in the 1889–90 pandemic. The 1918–19 pandemics, in terms of time required for epidemic spread, stands somewhere in between the other two pandemics considered here. The effect of increasing human mobility upon increasing the mobility of disease has never before been so dramatically demonstrated. Dozens of outbreaks in previously unaffected areas in the early weeks of the pandemic could be directly traced to passengers and crewmen of air and surface vessels recently arrived from epidemic regions.

As an epidemic disease, influenza was largely confined to the southern hemisphere during the winter and fall seasons of that half of the globe. With the arrival of fall in the northern hemisphere the epidemic disease shifted to the northern hemisphere. In 1889 there

was a similar suggestion of winter-fall involvement in the northern hemisphere with southward spread upon the arrival of fall in the southern hemisphere. From March or April on, however, the seasonal pattern was not as evident. In 1918–19 a northern-southern hemisphere seasonal pattern was less apparent for the world as a whole, although the greatest epidemics in Europe and North America took place in the fall and early winter. In the epidemics of inter-pandemic periods the seasonal pattern conforms fairly well. Epidemic influenza rarely occurs in the summer season of the respective hemispheres. Epidemics in Chile or Argentina are apt to occur in the fall and winter months of April through August, just as they occur between October and February in Europe and the United States. Season is often distinct from climate, of course, and factors other than weather, *per se*, are involved. For example, epidemics were reported in the United States with the arrival of the fall season regardless of the climatic character of the state. Arizona, Oregon, New York, Florida and Illinois experienced epidemics almost simultaneously. It is interesting to speculate that seasonal correlation of epidemic influenza is in some way related to the length of day, the factor which makes the word 'season' meaningful in many parts of the world. Although some studies of this nature have been in progress for several years, there is great need to further our understanding of varied host susceptibilities as they relate to physiological changes resulting from environmental differences in various seasons.

At least in the United States, the fall season also coincides with the opening of schools, and in 1957 school epidemics very often broke out (regardless of the geographic area of the school) within two to three weeks after the school opened for the semester. Schools in the United States are often crowded institutions and, as might be expected, experienced high attack rates. Crowding was everywhere incriminated as a major factor contributing to high attack rates, which were generally high in 1957 as they have been in all pandemics. Crowding also contributes to the speed of spread of the disease through an area but it does not affect the ultimate distribution of the disease. It became evident in 1957 that spread could occur very readily even in the absence of intense crowding. This is amply supported by analysis of the details of the international spread of the disease. Many isolated areas of low population density were affected as readily as the more populous regions, although they were usually not affected as early in the course of the pandemic.

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experience, also have fewer chances for infection due to decreased social mobility.

The disease was clinically mild wherever it appeared. Onset was usually sudden, and the three-to-five-day course was rarely complicated. Nevertheless, the 1957 pandemic had a conspicuous effect on mortality statistics. In many countries excess mortality due to influenza and pneumonia was measured with some precision. A reporting system incorporating 108 major cities in the Unites States provided weekly data for the U. S. Public Health Service. Excess mortality due to influenza and pneumonia began to rise at the beginning of October, peaked in 889 deaths for the week ending November 9, and declined to a nearly normal level (about 400 deaths per week at this season) by early December. Thus the epidemic was reflected by excess mortality for a period of about ten weeks for the nation as a whole. During this period the estimated number of influenza-associated deaths for the United States was in excess of 8,000. This excess may actually have been exceeded in several influenza epidemics experienced in the United States during the past several decades. This represents a considerable increase above seasonal expectancy but is only a small fraction of the total number of deaths which occurred in the United States as a result of the 1918–19 pandemic. During the same period the United States National Health Survey estimated that approximately 80,000,000 persons became ill during the epidemics. This represents a 50 per cent attack rate. For comparative purposes, it has been recorded that probably 50 per cent of the world's population had been ill by the end of the 1918–19 pandemic (Van Rooyen and Rhodes, 1948).

The age distribution curve of mortality was U-shaped—highest rates in young children and old persons, as in all previously studied epidemics or pandemics except that of 1918–19. In that pandemic there was a marked increase in young adult mortality so that a W-shaped curve of age specific fatality rates (Collins, 1931) resulted. Most complications have been pneumonic. Of the pneumonias the largest proportion have been pneumococcal, at least in the United States. Only about 10 per cent of reported influenza-associated pneumonia cases in the United States have been staphylococcal. The vast majority of the pneumonia deaths, however, have been in this relatively small staphylococcal pneumonia group. The pneumococcal pneumonias have, in general, responded very well to *antibiotic* therapy. A small number of sterile influenzal pneumonia deaths have

Just as in 1889, 1918, and in many lesser epidemics; military camps, children's camps, youth conferences, penal and medical institutions experienced consistently high attack rates. These close-contact groups were often affected well before the general population in the same area. Attack rates, *regardless of the type of population*, averaged higher than in inter-pandemic epidemics, doubtless a result of very high susceptibility of all populations in 1957. As in previous *pandemics, urban areas were generally involved before rural areas.*

Poverty could often be associated with high attack rates in 1957, and in similar fashion high incidence was experienced in certain *populations with social customs resulting in unusually large numbers* of close multipersonal contact.

As in previous epidemics and pandemics, no clear-cut important differences in male and female susceptibility were detected in 1957. Nor were any differences in racial susceptibility discovered although attack rates in a number of countries were higher in one racial or cultural group than another. In all such cases a readily apparent difference in economic or social conditions between the two groups could be offered as the likely explanation of the difference in attack rates.

Age-specific attack rates determined for several major epidemics in the United States closely resemble those for 1918 and other smaller epidemics. From maximum attack rates ranging from 50 to 75 per cent among children of school age the rates decline on the one hand to about 20 to 40 per cent in the pre-school age groups and on the other hand to 10 to 25 per cent in the adult age groups, with the lowest age-specific attack rate in the oldest adults. *It is probable* that both factors of close association (i. e. crowding in schools, school busses) and those of prior immunological experience (FRANCIS, 1953a; DAVENPORT ET AL., 1953) underlie these rates. *It seems likely that* elements of immunity may play the more important role among the adults, while factors of contact may be dominant in determining the attack rates for school-age and younger children. *If immunologic* prior experience were the only factor determining age-specific attack rates, the rates would be expected to be highest in the youngest children, and lowest in the oldest adults. The actual curve, however, while lowest among the oldest adults, is also low in the under-six age groups. This can be partially explained by the fewer opportunities for contact that are available to pre-school children than for children of school age. It should be pointed out, of course, that the oldest adults, who presumably have had the greatest amount of immunologic

that these characteristics include at least three factors; (1) a novel antigenic structure, (2) resistance to physical stresses encountered in transfer from person to person, and (3) virulence, *i. e.* ability to produce disease when infection is established. The dynamics of the interplay of these factors are by no means clear, but it is evident that antigenic differences alone are not sufficient to produce widespread disease. Other variants which have been isolated in recent years have been antigenically removed from the previously prevalent forms but large-scale epidemics did not occur. Some of these appeared to transfer readily because they were obtained from many parts of the world. In addition to the variability in antigenic composition among strains or virus isolates (JENSEN, 1957b) differences in resistance to inactivation by physical and chemical stresses have been noted (BRIODY, 1950). Attempts to compare the virulence of strains for laboratory animals and tissues are often recorded In this section we will discuss some of the observations made with these agents which antigenically form a new group within the type A, and have been designated as Asian or Far East.

A. Antigenic Relationships

One of the most remarkable achievements in modern preventive medicine was the prompt recognition of the viral isolates from Hong Kong and Singapore as being type A variants with immunologically significant differences (MEYER ET AL., 1957). These conclusions were reached with relatively few tests; confirmation came as millions of new cases developed. This can be seen as the logical and fruitful application of many advances in understanding of the antigenic relationships among influenza viruses (JENSEN, 1957b). The Promised Land of efficient control by immunization may not yet be in sight, but the early pioneers in this field must feel a sense of satisfaction and the novices are thrilling to the chase.

Even before the first vaccine trials many investigators realized the importance of acquiring fundamental information concerning the extent of antigenic variations among influenza viruses (FRANCIS AND MAGILL, 1938; SMITH AND ANDREWES, 1938). As is often the case with infectious agents, these viruses comprise several distinctly different antigenic groups and subgroups Each of the isolates obtained from influenza cases in humans and swine during the last 26 years contains

been reported, and an even smaller number of influenzal complications or influenza-related deaths have been due to myocarditis and encephalitis. Only one of the influenzal encephalitis deaths reported in the United States had been confirmed as due to the Asian strain by early December (McKee, 1957). Several others, however, were suspect on strong epidemiological grounds.

In the United States considerable evidence has been gathered (Langmuir et al., 1958; Dunn et al., 1957) on the pattern of spread of Asian strain influenza through the community. In general, the first reports from new communities referred to school children. Usually the high school children (13–18 years) were attacked first. Various explanations have been offered for this now well-documented fact but no completely convincing explanation has yet been forthcoming. A few days after the high school children first became ill in a given community, elementary school (children aged 6–13 years) absenteeism began to rise. First cases among the adult and pre-school groups apparently occurred soon thereafter, but the epidemic peaks for these groups followed those of the school children by one to two weeks. This impression is supported by data from an industrial absentee reporting system which was established by the U. S. Public Health Service in cooperation with industries in 36 cities of the United States. Industrial absenteeism rose one to two weeks after the onsets of school epidemics in many of the cities. Excess mortality was the last index of the epidemic to rise and fall. This lag is probably attributable to several factors among which could be: 1) the duration of illness before death, and 2) the time required for the disease to spread from the adult population to the infants and oldest members of the family, who are in general the individuals in any family with the smallest number of close daily contacts with other members of the family. At the same time they are most likely to develop a fatal complicating illness when attacked by influenza. It will be interesting to learn whether the same pattern of spread through the community was observed in other countries.

IV. Virology

The earliest epidemiologic and laboratory information indicated that the variant influenza viruses possessed all the characteristics necessary to produce a pandemic. It has been evident for some time

influence the course of an epidemic in vaccine trials (FRANCIS ET AL., 1947). Since that time data similar to those described for the PR8 set have been obtained with the type A viruses isolated during the years 1946–57. This set has been called A-prime because, although they were related to the older viruses, there was a major difference in antigenic constitution. Each of the A-prime isolates shared antigens with the result that vaccination with any of these strains often produced an antibody response in humans which was detectable with all members of the set. The FM1 strain isolated in 1947 (RASMUSSEN ET AL., 1948), is a prototype virus of this set (JENSEN, 1957c). This FM1 set of strains had been prevalent until early 1957. A majority of the world's population probably had been immunized in some manner with these agents because almost all sera examined recently contained detectable titers of antibody with the FM1 strain. The emergence of a new set of type A viruses was therefore a predictable event.

There have been many modifications of antigenic structure within the FM1 set. Isolates obtained during the years since 1948 from surveillance laboratories collaborating with the World Health Organization Influenza Program (PAYNE, 1954), have been examined at Influenza Centres in London and elsewhere with the purpose of detecting significant changes in antigenic structure. Isolates forwarded from these laboratories during 1950–51, 1953–55, and 1956–57 were conspicuously different when compared in cross tests with animal sera (ISAACS ET AL., 1952, JENSEN AND FRANCIS, 1953; HILLEMAN, 1954; JENSEN, 1957c). Comparisons of antibody responses in humans, however, indicated that antigenic differences of that magnitude were probably not of major immunologic significance, although the composition of vaccines was changed repeatedly to contain recent isolates (DAVENPORT AND HENNESSY, 1957).

A change was apparent early in 1956 when viruses obtained from Holland, Canada, the United States, England, India, Hawaii, and elsewhere were found to be different from strains circulated in previous years Members of this group were designated the "Dutch 56" viruses (ISAACS AND ANDREWES, 1956). They were obviously still within the FM1 set of type A strains. Epidemics which occurred in Japan during December, 1956 were caused by representatives of this group. Influenza cases were reported during January through April, 1957, in many parts of the United States, Europe, and Asia, and all isolates obtained were closely related antigenically to the Dutch 56

antigens which relate it to one of four types, termed A, B, C, and D. Antibody responses following infection or other antigenic experiences are type-specific; each type is unrelated immunologically to any of the other three groups. Among the strains of type A many differences of major or minor degree can be detected (JENSEN AND FRANCIS, 1953; JENSEN AND PETERSON, 1957). *Recently it has been suggested* (JENSEN, 1957a; ANDREWES, 1956) that what has occurred within the type A viruses could be likened unto variations on four themes. The new theme is heralded when a mutant with survival advantages *arises and becomes prevalent. Thus, four sets of type A viruses have* emerged thus far, each with antigenic composition sufficiently different from the preceding sets so that the antibody-prepared host population is not immune to the new variant.

Convincing serological evidence has been accumulated that strains of the swine set probably caused pandemic influenza in 1917–19 (LAIDLAW, 1935; SHOPE, 1936; DAVENPORT ET AL., 1953). Representative strains from this set possibly first appeared a few years before the 1918–19 pandemic, and it is evident that they were predominant until at least 1922–23. At that time a new set may have originated. Very few persons who began life after 1923 have a detectable antibody titer against the swine strains (DAVENPORT ET AL., 1955). Instead, many of the people born during the years 1923 to 1933 may have had their first experience with a strain closely related to the WS variant. The epidemic experiences in 1928–29 probably were due to members of the WS set of strains. The WS strain isolated in England in 1933 is somewhat antigenically removed from the type A strains recovered from patients in succeeding years (BURNET, 1937; JENSEN AND PETERSON, 1957). This was the first virus of human influenza to be studied and other agents from that era are not available. During the years 1934 to 1943 a large number of isolations were obtained, however, and although many variations were observed, all the viruses were antigenically within a set of which the PR8 strain can be considered prototypic. This fact was clearly demonstrated in the first experiments where protection was afforded by vaccination (FRANCIS, 1953b). Later delineation of the patterns of antibody in sera from various age groups supports this conclusion, as well as do the results of various antigenic analyses of strains (DAVENPORT ET AL., 1953; JENSEN AND FRANCIS, 1953).

Another major antigenic change among the A viruses came in 1946–47 and was recognized primarily by the failure of vaccination to

and recently epitomized by DAVENPORT and HENNESSY (1956, 1957) as the "Doctrine of Original Antigenic Sin." In brief, antibody responses are often conditioned by previous experience with antigens of related strains The antibody-forming mechanisms may be stimulated to increase the concentration of circulating antibody with the specific coding engendered by particular antigenic mosaics of viruses encountered many years before, perhaps during the first experience with influenza strains of that type Data from studies utilizing antibody-absorption from sera by various strains have supported this hypothesis (JENSEN ET AL., 1956).

One of the most interesting observations concerning the relationship of the Asian viruses to previous influenza viruses and first reported by MULDER (1957) is that sera from people who are 70 years or older often contain HI antibody against Asian isolates. Furthermore, anti-Asian titer increases have been found in sera from this age group after injections of type A vaccines which did not contain an Asian virus (MULDER, 1957; DAVENPORT, 1957; BOGER AND LIU, 1957) These reports are of particular interest because this is good evidence that viruses prevalent during the time of the 1889–90 pandemic contained antigens which are shared with these most recent A strains The incidence of HI antibody titers against Asian variants was less than one per cent in pre-epidemic sera from persons in the age range 0 to 70 years. There were a few younger people with specific antibody against this set, however, before it became prevalent this year. It is possible that they had experienced an antigenic stimulus with a variant which shared antigens with the Asian set viruses, but a more probable explanation is that these individuals had formed a less specific or more broadly reactive antibody than is the usual case in response to previous experiences.

B. Virus Isolation Methods

The isolation of the Asian variants from throat washings by amniotic inoculations of 11-day embryonate eggs was readily accomplished by laboratories with skill in these procedures. Several investigators reported better than 90 per cent virus recovery rates from patients demonstrated by serological responses to have been infected. On the other hand, negative results were often obtained by various laboratories with hundreds of specimens which had been

strains. The last isolates of this group obtained to date were recovered from a localized outbreak during June, 1957, in Canada.

Influenza occurred in China during late winter-early spring of 1957, but it was not until newspaper accounts were received in April of an epidemic in Singapore that action was initiated and isolates were obtained for study. Animal antisera prepared with a wide variety of influenza viruses did not react with the new isolates. Antisera from previous human cases did not neutralize the virus, but results of complement fixation tests identified the new agents as type A. Serum from animals immunized with several of the new isolates did not react with older viruses. Serum from people who had been repeatedly vaccinated recently with different type A variants likewise did not neutralize nor inhibit the new viruses. There was no doubt that a new set had appeared and preparations were initiated to follow the spread of these agents. At first the set was called Far East or Singapore 57, but by July the term Asian was generally agreed upon to designate this group of A influenza viruses. Since then, until the time of this writing (December) over 200 isolates have been sent to the International Influenza Center for the Americas and, with the exception of six type B strains, all were closely related to the first strain studied, A/Asian/Japan/305/57. The FM1 set apparently has been completely replaced by the Asian set just as the PR8 set disappeared when the FM1 set began its period of prevalence.

The antigenic relationships of Asian strains to earlier forms of type A are most definitely illustrated from results of complement fixation tests with convalescent phase serum from humans or ferrets. The reaction can be demonstrated either with extracts of infected tissues containing "soluble antigens" or with the viral antigens in fluids from infected embryonate eggs or tissue cultures. Even with the more specific hemagglutination-inhibition (HI) technique, cross reactions may be seen with certain human sera. In routine serologic tests to detect infections with Asian strain influenza many laboratories have reported a diagnostic rise in antibody titers measured in HI tests with older strains such as A/Denver/1/57 or PR8/34 with or without a comparable increase observed against the Asian strain. These reactions were obtained with serum specimens from patients whose throat washings had yielded Asian-like viruses. It must be assumed, therefore, that these patients produced antibody which may be more readily detected with an older strain than with the recent variant. The phenomenon is similar to that previously observed

hemagglutinin titrations. Hemadsorption apparently provides a more sensitive testing procedure for influenza and might be developed for application to other virological problems.

Monkey kidney cultures have been demonstrated to be of value in studies with influenza viruses (TAKEMOTO ET AL., 1955; MOCABGAB ET AL., 1955; HENRY AND YOUNGNER, 1957). There is some question yet as to the relative efficiency of the chick embryo and monkey kidney systems as isolation media for influenza viruses. MOCABGAB has reported (1957) satisfactory results with Asian strain antigens produced in monkey kidney cultures for HI and CF techniques.

Several investigators have been interested in seeking more rapid means of diagnosis than the two to six days routinely required by isolation of the virus in eggs. Detection of CF antigens in infected tissues can occur within the first 24 hours after inoculation, but it is not often attempted. Smears from nasal swabs and washings will give specific staining with fluorescein-labelled antibody (LIU, 1956), and this procedure, as well as that described by GOLDWASSER AND SHEPARD (1958) with stained complement are currently being evaluated in several laboratories as to their efficacy in diagnosis of influenza

C. *Serology*

Quantitation of the number of cases of influenza in an epidemic is dependent upon data developed as a result of laboratory examinations of materials obtained from patients. Procedures which are necessary for virus isolation are expensive and time-consuming so that methods designed to measure the incidence of antigenic experience are superior when diagnosis of infection is to be attempted with a large number of patients. Routine serologic tests for influenza are the hemagglutination-inhibition (HI) and complement fixation (CF) methods. Although standard procedures have been described (Committee on Standard Serological Procedures in Influenza Studies 1950, WHO Expert Committee on Influenza 1953; JENSEN, 1956), many variations and modifications are employed, particularly with the HI test.

Antibody which combines with the viral particles to prevent agglutination of erythrocytes by the virus can also be measured in the antigen-antibody union which fixes complement. There are, however, other antigen-antibody complexes detected in the CF test which are not apparent in HI tests. These conclusions are drawn

improperly obtained or not shipped under ideal conditions. Many of the suspect materials must have been from patients suffering from the non-influenzal diseases which are so difficult to differentiate from influenza on clinical grounds (DINGLE AND FELLER, 1956). The difficulties encountered were typical of those which ordinarily attend attempts at influenza virus isolations. Often amniotic fluids from injected embryos did not contain sufficient concentrations of virus to permit detection with erythrocyte suspensions, but when these fluids were inoculated into a second group of eggs, the hemagglutinins could be demonstrated. Many isolates agglutinated red blood cells from guinea pigs and humans more readily than those from chickens in the first few transfers in eggs in a manner similar to that frequently observed in the past with type A viruses (BURNET AND BULL, 1943; BRIODY, 1950).

One of the most interesting innovations described during this epidemic is the "Adsorption-hemagglutination test for influenza virus in monkey kidney tissue culture" reported by VOGEL AND SHELOKOV (1957). These investigators have found that monkey kidney cultures grown in monolayers and maintained in tubes in the usual media often support the propagation of influenza viruses from throat washings. Their method of detection is unique because the phenomenon of hemadsorption is utilized. Erythrocytes from guinea pigs, chickens, sheep, monkeys, and humans have been tested, and the guinea pig cells appear to be most satisfactory. The reaction is viewed under low power of a microscope. When positive, characteristic patterns are formed with the red blood cells firmly attached to the cell culture sheets. It is very striking to find individual tissue cells with "rosettes" of the erythrocytes, and chains of erythrocytes may be formed near groups of tissue cells. This reaction can be demonstrated in some cases as early as 24 hours after inoculation of the tissue cultures. Tubes which are negative at that time can be incubated for several more days with the red blood cell suspensions and periodically examined. When the positive reaction is demonstrated, the cell sheets are harvested and homogenized for transfer of the agent into additional cultures. Serologic typing of the viruses can be accomplished by specific inhibition of hemadsorption with immune sera. The method is being evaluated in several laboratories and promising results have been reported. Often cytopathic effects of the influenza viruses are difficult to detect in tissue cultures and the production of hemagglutination can be too limited to permit usual

Vibrio cholerae cultures (VAN DER VEEN AND MULDER, 1950), tryptic digestion (SAMPAIO AND ISAACS, 1953), and oxidation with periodate solutions (BURNET AND LIND, 1954). Several different non-specific inhibiting substances are found in sera, and the evidence indicates that they are mucopolysaccharides and mucoproteins. Treatment of sera with vibrio filtrates (sometimes referred to as RDE or receptor destroying enzyme) or trypsin will result in the destruction of inhibitors (mucoprotein) which combine with many strains, but these methods have not been of value in handling the problem with non-specific inhibitors which unite with the Asian isolates.

In current experiences, satisfactory results have been obtained with a periodate method in removing non-specific inhibitory materials from ferret, chicken, and human sera. The "animal-line" strains of the Asian set were universally susceptible to the inhibitors which can be destroyed by periodate treatment. The method which has been recommended is a modification of that first described (BURNET AND LIND, 1954), and consists of mixing one volume of serum with two volumes of M/90 potassium periodate solution in distilled water. This mixture is held at 4° C overnight (16 to 18 hours). It is important that the periodate solution be freshly prepared. At the end of the incubation period, a volume of one per cent glycerol-saline (pH 7.2) equal to that of the periodate volume is then added to reduce the excess periodate. This technique has been routinely successful in many laboratories and does not affect the titer of specific antibody (ROBINSON ET AL., 1957).

The methods currently employed at the Influenza Center for the Americas and recommended to collaborating laboratories and other state and diagnostic service agencies, are essentially as outlined previously (JENSEN, 1956). A standard HI test procedure for laboratories in the Americas is to test varying dilutions of serum with four hemagglutinating units of virus, allowing 30 minutes for incubation at room temperature before adding 0.5 per cent suspensions of chicken erythrocytes. The volumes ordinarily employed are 0.25 ml for the serum and for the virus suspension and 0.5 ml of the red cells. Tests are read after 45–60 minutes at room temperature during which time the cells settle in characteristic patterns, and the greatest dilution of serum which causes inhibition of hemagglutination is taken as the endpoint and titer of the antibody in the serum. The complement fixation procedure followed uses two optimal units of antigen and complement with an overnight fixation period at 4° C.

from observations that the antibody response is more specific in HI tests than in CF tests, and in some cases significant increases in antibody can be detected with the CF test which are not demonstrable with the HI technique. This raises the question as to whether variation among strains, in their affinity for antibody, may not account for some of these differences. It is well known that the activities of some isolates are more readily neutralized by specific antibody than are other viruses which are closely related antigenically (HIRST, 1943; VAN DER VEEN AND MULDER, 1950; ISAACS ET AL., 1952; MULDER ET AL., 1956). Work with the viruses first isolated during the Asian epidemics made it clear that some did not measure antibody responses in HI or *in ovo* neutralization tests following convalescence from the disease while significant differences in antibody titers were seen with the paired sera in CF tests with the same viruses. With other viruses from the same epidemic the production of antibody could be demonstrated. The phenomenon was first called P-Q phase variation by VAN DER VEEN AND MULDER (1950) and has been under investigation for some time. Extreme examples of Q-phase condition were found with some isolates. Sera from ferrets or humans who had been infected recently with those variants showed an antibody response measurable in HI tests with related strains but not with the infecting strain. When these viruses were transferred serially in ferrets and mice and subsequently in eggs, the strange antigenic behavior is lost (ISAACS ET AL., 1952; JENSEN ET AL., 1957a). Several of the viruses obtained from the Asian epidemic have been so manipulated in the laboratory to eliminate the Q-phase condition. One of these, the A/Asian/Japan/305/57 strain, after transfers through four eggs, one ferret, three mice, and then in several additional eggs, was shown to be over 60 per cent more efficient in HI test diagnosis of cases than the virus which had been passed in only a few eggs. The animal line of Japan/305 was adopted as a standard Asian virus for production and distribution of diagnostic reagents from the International Influenza Center for the Americas.

Another vagary of the hemagglutination-inhibition test is that of non-specific inhibition by materials in sera which combine so effectively with certain isolates that serotyping identification can not be carried out. Diagnostic rises in specific antibody concentrations are obscured (VAN DER VEEN AND MULDER, 1950; HILLEMAN AND WERNER, 1953; JORDAN AND OSEASOHN, 1954). It is necessary to find means of removal or destruction of these inhibitors and three methods have had routine use. These are: digestion with filtrates of

With a group of 496 cases diagnosed by either method, it was found that the CF test more frequently led to a diagnosis than did the HI technique. In only 260 cases were the results of both tests positive. As it will be noted from inspection of the data, only one or the other of the tests was positive in the remaining 236 cases and this was more often the CF method. A group of investigators directed by HOUSER (1957) has reported a similar observation and their data indicated that some of the differences might be attributable to variation in the period of time elapsed between the collection of the first and second serum specimens, and that the antibody detected by CF tests probably increased in some cases more rapidly or earlier than that measured in HI tests. This suggestion is being investigated further in several laboratories.

V. Sero-Epidemiology

In every epidemic there are individuals who experience infections without evident disease. Many projects have been initiated therefore to learn the effect of the passing epidemics on antibody patterns in different age groups (HENNESSY ET AL., 1955). Hypotheses and concepts which many investigators are testing were formed from results of studies combining the serological and epidemiological procedures. One of the questions foremost in the minds of many people in the field of public health concerns the incidence of clinically inapparent infections during the 1957 pandemic. Preliminary data are already available from a few sources.

During August 1957 a major community-wide epidemic of Asian strain influenza occurred in the United States in Tangipahoa Parish, Louisiana. This was the first Asian strain-epidemic to involve the general population in North America. It is of interest that schools were open at the time of the epidemic in this parish while they were closed in most other parts of the country. An extensive epidemiologic study was initiated, the results of which have not yet been published (DUNN ET AL., 1958). As a part of this study surveys were made during the fall to obtain information on the serological attack rates for comparison with the attack rates recorded by epidemiologic techniques (CAREY ET AL., 1958). The largest samples were obtained among children of high school (13–20 years) and elementary school (8–14 years) age.

before addition of the hemolytic system. Endpoints and titers are read as the greatest dilution of serum which results in fixation of complement so that not more than an estimated 25 per cent of the sensitized sheep red cells are lysed. For this test veronal buffer is the diluent and volumes of 0.1 ml are used of antigen and serum, 0.2 ml complement, and 0.2 ml sensitized sheep cells. Antigens for use in both HI and CF tests which have been prepared and distributed to requesting laboratories were allantoic fluids harvested from embryonate eggs infected with one of the following strains: A/Asian/Japan/305/57, A/Denver/1/57, and B/GL/1739/54. The Denver/57 is an isolate from a February epidemic in Colorado, U. S. A. (MEIKLEJOHN, 1956), and closely related antigenically to the Dutch-56 viruses, and the B/GL strain, isolated at Great Lakes, Illinois in 1954 (SEAL, 1954) is prototypic of all recent type B isolates. Over 30 liters of these materials were dispatched in the early months of the epidemic in the U. S. A. Typing sera also distributed for use in HI tests with isolates obtained in various laboratories were prepared in adult chickens by intravenous and intraperitoneal injections of allantoic fluids from infected eggs.

Results of serologic tests have been reported to the Center by many of the State Health Laboratories and other investigators collaborating with the World Health Organization influenza program (JENSEN AND HOGAN, 1958). It was of interest to learn the comparative efficiencies of the HI and CF tests as compiled from the data submitted where both methods had been used to test pairs of sera. The information available at this writing is summarized in table I.

Table I. Comparative Rates of Serologic Diagnosis* by Complement Fixation or Hemagglutination Inhibition Tests

Test Method	*Number*	*Per cent*
Total cases diagnosed by either test	496	100.0
Total cases diagnosed by complement fixation	439	88.5
Total cases diagnosed by hemagglutination-inhibition	317	64.0
Cases positive by both tests	260	52.4
Cases positive by complement fixation only	179	36.0
Cases positive by hemagglutination-inhibition only	57	11.5

* Convalescent phase serum containing at least four times as much antibody as found in the acute phase serum.

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Sera were obtained two to three months after the epidemics occurred. Tests were carried out by the hemagglutination-inhibition test previously described. The *distribution of antibody titers* in sera from 298 high school children is presented in table II. Almost identical results were obtained with sera from 131 elementary school children. From examination of these data it can be seen that at least 88 per cent of those in the sample considered to have had clinical influenza by the simple questioning technique used, had actually been infected by the Asian strain virus. The samples for the two school age groups have been shown to be representative of the larger school populations sampled in the epidemiologic study. It has thus become apparent that *the very high attack rates among* school children in the parish (60–70% or more, depending upon the school) recorded in the epidemiologic survey were probably accurate. The serological data show, furthermore, that subclinical infection, while present, was much less common than clinical infection (Table III).

Table II Distribution of Hemagglutination-Inhibition Antibody Titers in Sera from 298 High School Children

Titer with Asian/Japan/305	Illness (Total 191)		No Illness (Total 107)	
	Number	Per Cent	Number	Per Cent
0	21	11 0	52	49.6
5	2	1.0	0	0 0
10	7	3 7	3	2.8
20	42	22 2	18	16 8
40	65	34.0	21	19.7
80	44	23.0	12	11.2
160	10	5.3	1	0.9
320	0	0.0	0	0 0

Table III. Summary of Sero-Epidemiologic Experience of Tangipahoa Parish High School Children (Total 298)

Influenza Detection Rates Method	Infected	Not Infected	Per Cent Infected	*Serologically Detected Infections* (Total 223)	Number	Per Cent
Serologically	223	75	75 0	Clinical	168	75.3
Epidemiologically	191	107	64 2	Subclinical	55	24 7

In the group which had not reported an illness there were approximately 50 per cent who had been infected as shown by specific antibody titers. Distribution curves of antibody titers for those with subclinical and clinical illness were virtually identical. Among those with significant antibody concentrations after the epidemic a subclinical infection rate of approximately 25 per cent was found. Only 25 per cent of the total population sampled had not developed antibody detectable by this test.

These results are believed to be representative of experiences in comparable age groups in the United States. Additional studies are underway with complete sampling of communities in an effort to determine rates of antigenic experiences and illnesses in all age groups. It is of interest to point out that the ratio of clinically apparent infections was much higher in this study than in those previously measured. For example, it was shown by the Commission on Acute Respiratory Diseases (1948) at Fort Bragg, North Carolina in a type A epidemic in 1943 that approximately 79 per cent of serologically proven infections were clinically inapparent. This differs significantly from the 25 per cent inapparent infection rate found in the high school children this year. Undoubtedly the antibody in many of the acute phase sera of soldiers in 1943 influenced the clinical course of the infections. Some observations relating to virulence of epidemic influenza have been reported, but the principles underlying the phenomena have not yet been elucidated (Francis, 1953a).

VI. Vaccine

The immediate reaction in public health quarters in the U. S. and elsewhere to news of an approaching influenza epidemic with unknown virulence potential was to initiate steps necessary to provide specific immunity by vaccination. The rationale was very clear; there are no other effective means of control; no specific therapy for cases has been demonstrated of value and there have been 15 years of fruitful vaccine field trials conducted by the Commission on Influenza under the auspices of the U. S. Armed Forces Epidemiologic Board, as well as studies by other groups in England and elsewhere (Francis, 1953). Early reports from authorities in the Far East indicated that personnel recently vaccinated with the old formula vaccines apparently had not derived any protective effect when challenged by this year's

epidemic situation. This was very reminiscent of the 1947–48 experience when vaccination did not noticeably affect the course of the first FM1 set epidemic (FRANCIS, 1953b), because the vaccine did not contain sufficient quantities of antigens dominant in the new set. There was need, therefore, to utilize one of the new antigenic variants obtained from the Asian epidemics in new vaccines.

Rapidly formed opinion in the U. S. A. concluded that vaccines should be produced as quickly as possible so that these biologics could be used on a scale never before attempted; the incidence of antibody against this variant was extremely low in the general populations. The task was to obtain larger quantities than ever before so that 60 million doses might be available in a few months. A very dramatic race thus developed between production and use of this vaccine and the advent of widespread epidemics in this country.

A. Production

In the United States six pharmaceutical companies are licensed to market influenza vaccines and these companies began pilot studies upon receipt of the new variant late in May. Although certain trade secrets are kept by these companies concerning various manufacturing processes, the basic methods are similar and each product must meet specifications established by the Division of Biologics Standards of the U. S. Public Health Service. Minimal potency standards are set. Furthermore, each lot must pass requirements of bacteriologic and toxic safety, and no infectivity should remain in the viral suspensions as determined by inoculations of embryonate eggs. There is some question as to the importance of this latter specification as a safety precaution because it is generally believed that influenza illnesses do not result from the parenteral injections of fully active virus.

The greatest problem in all cases is to obtain good concentrations of virus in allantoic fluids pooled from eggs infected with new isolates. It was found universally that very low titers were obtained with the first Asian isolates. Various manipulations were tried in research departments of the companies in efforts to increase the yields, including rapid transfers of the agents in eggs, incubation of the eggs at different temperatures and for various periods of time, use of eggs from different breeds of chickens, and treatment of the eggs with cortisone (KILBOURNE, 1957). All resulted in no significant improve-

ment. The concentrations of virus obtained in production pools were only one-tenth or less of those found with old stock strains. Later in the year other variants were found which gave much better results and after multiple transfer the progeny of the first isolates were developed as satisfactory production strains. Although the manufacturers are not required to indicate which strain of virus of the Asian set is used to produce the vaccine, it is known that the A/Asian Japan/305/57, A/Asian/Formosa/313/57, and A/Asian/Singapore/1/57 are most commonly employed.

The several methods used to concentrate virus from infected fluids include among others; adsorption-elution from erythrocytes, differential centrifugation, precipitation with protamine and aluminum phosphate. Sedimentation of the virus by high speed centrifugation is probably the most generally used method of those publicized. There is always an effort to purify the viral suspensions so that less egg proteins and non-viral materials are contained in the vaccines and thus decrease pyrogenicity and other undesirable reactions. Two methods are used to destroy infectivity without great loss of antigenic potency, namely by treatment with formalin or ultra-violet light.

The potency of influenza vaccines in the final analysis is determined by the efficiency demonstrated in reduction of cases in vaccinated humans as compared to the number observed in strictly comparable groups of nonvaccinated persons. There must be other means of appraising the antigenic potency of various preparations before large-scale use, however, and the estimation of virus particle concentration appears to be an index of the quantity of viral antigen. Here again the capacity of influenza viruses to agglutinate erythrocytes is utilized to determine virus concentration because this function is not drastically impaired by methods which destroy infectivity. The method described by MILLER AND STANLEY (1944) has been adopted as a procedure with a degree of precision for determining the hemagglutinating activity of virus suspensions Results are expressed in terms of chicken cell agglutinating (CCA) units per ml. Upon consultations with representatives of the vaccine producers during the second week in June, it was learned that the virus yield was not as great as usually obtained, and therefore it would be difficult to produce vaccine with more than 200 CCA units per ml. Harvested fluids contained only 10 to 90 CCA as compared to the 300 or greater which were usually obtained with stock strains. It was agreed that a 200 CCA minimum requirement would be acceptable until more potent

materials could be prepared, at which time the specifications would be raised to approach the 500 to 1000 CCA vaccines which have been successfully used during the past in civilian and military groups in the U. S. A. Although the Division of Biologics Standards had required in the past that protocols submitted with each lot of vaccine also include potency tests in mice, such tests could not be carried out on the newly prepared Asian vaccine if the material was to be used in time, and the new vaccine was released on CCA determinations alone.

During June and July the first experimental lots of monovalent (one strain) vaccine at the 200 CCA units per ml level were made available for studies in human volunteers. Approximately 4 million doses (1 ml given subcutaneously) were produced and released for use in August, 9 million in September, 17 million in October, and 8 million in November. Some manufacturers combined Asian strains vaccine with that produced with older type A viruses and a type B strain, and almost 5 million doses of these polyvalent (several strains) vaccines were released during this time. By the end of September the manufacturers reported that much greater yields could then be obtained and a new potency level of 400 CCA for monovalent vaccines was set to become effective the first of December. During November over 9 million doses at the 400 CCA concentration were made available. In total, approximately 52 million ml of vaccine containing the Asian variants were marketed during the four months of August through November.

B. Distribution and Priorities.

In early planning meetings it was recognized that the demand for vaccine would far exceed the supply for many months. The question of controlled distribution and establishment of priority groups within the population was discussed at length. There were two basic considerations in all these deliberations arising from the unknown virulence factors in pandemic influenza. One problem was the public health aspect of a mild disease with high morbidity rates sweeping rapidly through the country; the other concerned the need to prevent increased mortality rates associated with similar epidemics in the past. In the first place, with the strong possibility of high attack rates with resulting disruption of normal community life, an effort should be made to immunize certain personnel such as those respon-

sible for medical services, protection of the community or communication and transportation. From a different point of view, individuals who are classified as poor medical risks, such as patients with cardiac or chronic pulmonary disease who might more frequently die from relatively mild infectious disease, should be among the first to receive vaccination The consensus of opinion was that governmental regulation should be avoided unless absolutely necessary and that the usual doctor-patient relationship be maintained. Medical societies and state and local health authorities were advised concerning the use of the vaccine. The pharmaceutical companies voluntarily agreed to divide supplies of vaccine as they became available, in order that requests might be filled on an equitable basis among the states.

VII. Vaccine Evaluation Studies

Although many facets of influenza prophylaxis by immunization with killed vaccines have been carefully evaluated during the last fifteen years, such great interest was stimulated this year that at least 56 studies by 33 investigating groups in the United States were initiated during the peidemic. These were reported in a survey conducted by the Public Health Service Influenza Research Committee (COLE, 1957). Several large studies are in progress in the Armed Forces (DAVENPORT, 1958), and industries, as well as a number on a smaller scale at universities or conducted by the Public Health Service and State Health Departments. At least 100,000 persons of all age groups are represented in the studies. Special experiments involve immunization of egg sensitive patients, pregnant women, infants, aged adults, and persons with chronic pulmonary disease. Unfortunately, not all of the trials include adequate controls, but observations of lasting value will surely be made in several experiments with superior design. In the main, the questions being asked concern optimal dose of vaccine and method of administration to produce effective reduction in number of cases of influenza or most efficient development of antibody. Some data have already been collected and will be discussed in this section Details of the experiments will not be given, however, pending publication of completed studies by the individual investigators in appropriate journals.

A. Stimulation of Antibody Production.

The correlation between antibody titers (as determined by neutralization and HI tests) and immunity is such that the incidence of influenza is greatly reduced in those individuals who possess higher concentrations of circulating antibody. This does not mean that in the absence of detectable antibody there is no immunity nor that the development of specific antibody always provides protection. The trend, however, is obvious, and measurements of antibody stimulation have often served well to indicate the effectiveness of immunization procedures (FRANCIS, 1953b; SALK, 1952; DAVENPORT AND HENNESSY, 1957). Antigenic potency tests in humans with the new Asian strain vaccines were therefore indicated as a first step in evaluating their usefulness. It was recognized that several unusual features might influence results obtained with this vaccine. The Asian set viruses were so different that vaccination would be the primary antigenic experience in a vast majority of populations. This condition was quite unlike that found in recent experiments where vaccines contained antigens closely related to the immunologic foundation formed from previous antigenic experiences (DAVENPORT AND HENNESSY, 1956, 1957; JENSEN ET AL., 1956). Furthermore, the viruses used as seed for the production of vaccine antigen had not undergone extensive laboratory manipulation and transfers in animals, as had the older vaccine strains.

The amount of virus as determined by CCA values contained in the new vaccines was less than optimal. Nevertheless, results of animal immunizations indicated that considerably more antigen was contained in the preparations than the CCA titer suggested. Several experiments were designed, therefore, to test with even less than 200 CCA. Tests with the first available lot were carried out in adults in the age range 18 to 50 years using one ml intramuscular injections of 50, 100 and 200 CCA units (JENSEN ET AL., 1957b). The antibody responses as determined by HI tests, using sera from approximately 60 subjects in each group, are summarized in table IV. The hemagglutinin used was the Asian/Japan/305/57 strain after transfers in animals. The sera had been treated with periodate to remove nonspecific inhibitors as described in a previous section. Titers are [illegible] of other reagents.
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[illegible] ated

Table IV. Incidence of Antibody Production in Adults* Injected Subcutaneously with Three Concentrations of Monovalent Asian Vaccine

Vaccine CCA	Response After One Dose Per Cent with Titers		Response After Two Doses Per Cent with Titers	
	10** or greater	40 or greater	10 or greater	40 or greater
50	35.4	4.2	73.9	43.5
100	40.7	9.3	81.6	38.8
200	58.8	13.7	81.1	30.2

* Tests with approximately 50 per group.

** Titers are expressed as reciprocals of the greatest dilution of serum (before addition of other reagents) which inhibited 4 hemagglutinating units of the Asian/Japan/305/57 E4 F1 M3 E10 virus. Sera were treated with periodate to remove inhibitors.

virus concentration. Sera were also obtained at the one, two, four and six week intervals. The initial response was not detectable at one week except in one or two individuals, and titers were diminishing after four weeks. The volunteers were revaccinated at the four week interval and bled again two weeks later. As can be seen from inspection of these results, production of antibody was more often detected in higher titer when the greater amounts of virus were injected. Serum obtained after a second dose of vaccine contained H. I. antibody twice as often as did the specimens following the first dose. The proportion of subjects producing antibody was practically the same regardless of the virus concentration injected in the first dose. It was therefore evident that antibody production could be stimulated in humans with this vaccine and that a second dose would result in a much higher incidence of antibody production.

Data obtained from CF tests with sera collected after the first dose are summarized in table V. The proportion of each group which responded with four-fold or greater increases of CF antibody are presented. Some degree of specific antigenic response was noticed because many more rising antibody titers were detected with the Asian strain than with the PR8 or FM1 viruses. The soluble antigen prepared from extracts of chorioallantoic membranes from eggs infected with the Japan/305 virus often measured antibody increases, and at rates comparable to those obtained with the viral particles in allantoic fluids. There is reason to doubt the usefulness of this antigen

Table V. Per cent of Subjects* with Antibody Responses to Vaccination as Measured in Complement Fixation Tests

	Per Cent Responses with Type A Antigens			
Vaccine CCA	Total with Any Antigen	Asian Viral	Asian Soluble	PR8 or FM1 Viral
50	48.2	30.4	23.2	17.9
100	43.1	24.1	31.0	19 0
200	73 7	61.4	54 4	12.3

* Approximately 60 adults per group. Sera obtained 2 weeks after 1.0 cc subcutaneous infections of a lot of Japan/305 vaccine.

Only 4-fold increases in antibody titers were considered significant and are here tabulated.

for differentiation between post-vaccination responses and antibody developed in convalescence (HENLE, 1953; ROSENBAUM AND WOOLRIDGE, 1956). Results of tests with control antigens (allantoic fluids and membrane extracts from non-infected eggs as well as from eggs infected with type B strains) made it clear that the responses were influenza type-specific. Again the per cent developing antibody was greater in the group which had received the 200 CCA vaccine. A surprising observation was that titers in these cases were not heightened following the second dose. For some reason the great majority of each group who were to produce CF antibody increases did so with one dose and titers were not increased after the second injection. This is very different from the results obtained using the HI technique and serves to emphasize the importance of further investigations concerning the relationship between CF and HI antibody and immune responses.

Several additional lots of vaccine prepared with the Japan/305 seed by various methods were tested in other groups of adults, to compare again the responses obtained at the 50, 100, or 200 CCA levels. Results presented from the first experiment are representative of those found in these additional studies (JENSEN ET AL., 1957b). There was considerable variation among lots of vaccine as to their antigenic potency although adjustments were made so that each contained a standard CCA value. It was evident that some lots, diluted to contain only 50 CCA, produced responses superior to those

found with four times as much virus in other lots. One of the best results demonstrated was with some vaccine prepared with the Asian/Formosa/313/57 seed. Aliquots of this vaccine had also been adsorbed to aluminum phosphate. No advantage was noted with this material because the incidence of antibody titers measured after 6 weeks was not greater than that obtained in groups which had received the same amount of virus suspended in saline. In all of these experiments a trend toward better antibody production was seen with the increasing concentrations of virus (JENSEN ET AL., 1957c). In the next, vaccine containing 500 CCA units per ml was employed (DULL ET AL., 1957). From the summary of results obtained from this experiment presented in table VI it will be seen that 500 CCA units elicited antibody significantly more often than did lesser amounts of virus. Observations by DAVENPORT (1957) and HILLEMAN (1957) support the conclusion that superior antibody responses could be obtained with higher CCA levels and that the CCA value for monovalent vaccines of this type should be greater than 200 CCA units per ml. As mentioned previously in this report, the standard was raised to 400 CCA by the U. S. Division of Biologics Standards (MURRAY, 1957).

Because the vaccine was in short supply, many physicians attempted to immunize with intracutaneous injections of 0.1 ml despite the fact that there were virtually no data concerning protection afforded by vaccinating in this manner. A series of experiments has since been completed (JENSEN ET AL., 1957d), the results of which indicate that the magnitude of antibody responses is exactly that

Table VI. Incidence of Antibody Responses* to a Standard** Monovalent Asian Strain Vaccine

Vaccine CCA	Per Cent Response by Titers 10 or greater	40 or greater
50	48 3	26 7
200	66 1	40 3
500	83 6	50.8

* H. I tests with sera from 50 adults vaccinated 2 weeks previously.

** Standard lot of vaccine furnished by Division of Biologics Standards, U.S.P.H.S.

which is obtained when similar amounts of this antigen are injected subcutaneously. With only one-tenth as much antigen being used in this procedure as when one ml is given subcutaneously, the number of people responding is diminished, but the relationship is not linear. Antibody responses have been studied in school children (6 to 14 years) and adults. In two experiments arranged to define the differences to be noted when 20 CCA units had been given intracutaneously or 100 or 200 CCA units injected subcutaneously, the rate of response was one-half to one-third greater with the larger doses. Comparable data have since been reported by BOGER AND LIU (1957). In order to investigate the matter further, groups of 50 adults were injected intracutaneously with 0.1 ml of various dilutions of vaccine which originally contained 1,000 CCA units per ml (JENSEN ET AL., 1957d). The distribution of HI antibody titers demonstrated in sera two weeks after one or two doses is shown in table VII. Although one group received only 12.5 CCA units, responses occurred just 16 per cent more often in those who had been injected with 100 CCA units. The data from tests after 2 doses indicated an even smaller difference in the total number of significant antibody increases. Comparisons of the geometric mean titers indicate that higher concentrations of antibody were more often educed with the greater amount of virus.

Table VII. Distribution of Hemagglutination-Inhibition Antibody Titers After Intradermal* Vaccinations

	After 1 Dose Per Cent Response by Titer		
CCA Injected	10 or greater	40 or greater	Geometric Mean Titer
12.5	34 0	4 3	2.4
25	37.0	15 2	3.4
50	44 0	13.7	4.1
100	50 0	28 0	6.3
	After 2 doses		
12.5	68.2	43.1	11 3
25	65 0	42 5	10.8
50	73.5	51.0	18.9
100	75 0	54.2	22.5

* Groups of 50 adults injected with 0.1 ml at an interval of two weeks.

The trend toward superior responses found with larger CCA values in vaccine injected subcutaneously is echoed by these experiments. We cannot leave a discussion of these data without pointing out that two injections given 2 weeks apart with 50 to 100 CCA material have been demonstrated to stimulate good antibody production. Results, in terms of frequency and concentration, were similar to those found after one injection of the most concentrated (500 CCA) preparation tested. Additional details and discussions of these experiments will appear in future publications of completed studies. Brief communications from other groups of investigators (SIGEL ET AL., 1957; BOGER AND LIU, 1957) have recently appeared which provide support for conclusions drawn in this section. A complete review of all vaccination studies instigated this year is warranted in the near future.

B. Incidence of Clinical Reactions to Vaccine.

Despite military experience to the contrary, rumors were circulated in the U. S. A. concerning a high incidence of febrile reactions to the vaccine. Although one lot of the first batches tested for the production of antibody had caused moderate reactions in many of the subjects, that particular preparation had been concentrated by the adsorption-elution from erythrocytes technique and was quite opalescent. The usual experience with early lots was that very few individuals noticed any marked reaction. In none of the experiments reported by other investigators have serious reactions occurred and the moderate reactions described were brief, usually lasting less than 24 hours. From one of the best studies of this problem, SARTWELL AND SELTSER (1957) reported that almost as many control subjects (placebos of formalized saline) as vaccinated (250 CCA) complained of contitutional symptoms. In these groups which totaled 2,127 in ages ranging from 1 to over 40 years, there was an incidence of systemic reaction of 10.5 per cent in the controls and 12.7 per cent in the vaccinated subjects. It is clear, however, that children more often developed a febrile response than did adults, even when smaller doses were given (QUILLIGAN ET AL., 1949). Data derived from an experiment with families of Public Health Service Officers at the Communicable Disease Center illustrate this point These are presented in table VIII. Fever was very transient as only eight persons reported elevated temperatures on the day following vaccination.

Table VIII. Incidence of Febrile Reactions to a Lot of Influenza Vaccine (200 CCA Units per cc).

Age Group	Vaccine	*Number*	*Per Cent with Fever**
0–4	0.1 cc	75	5.3
5–9	0 5 cc	88	26 0
10–19	1.0 cc	90	13.3
20	1 0 cc	142	2 1

* Temperatures of 101°F. or greater recorded the evening after vaccination.

When 93 of the children were revaccinated one week later, only 2 developed a fever.

More recently tests were initiated with several lots of 400 CCA vaccine in college students (JENSEN ET AL., 1957b). The proportion of clinical reactions to these materials in a total of 957 subjects is shown in table IX. In this case the saline placebo did not have formalin added

Table IX Per Cent of Clinical Reactions to Vaccination with Varied Concentrations of Influenza Viruses

	Subcutaneous Injection of 1 0 cc			
Vaccine CCA/cc	Pharmaceutical Company	Slight*	Moderate	Negative
200	3	13.4	3.1	83 5
400	1	34 4	3.7	61 9
400	2	25 0	6 2	68 8
400	3	21 5	0	78.5
400	4	27.7	4 8	67.5
400	5	32 6	3 3	64 1
*700 poly.***	1	26 0	3.1	70 8
Saline	–	13.7	5 0	81.3
	Intracutaneous Injection of 0.1 ml			
400	1	13 7	3 7	82.6
400	2	13 7	2 5	83 8
700 poly.**	1	16 2	0	83.8

* Slight = sore arm or headache, Moderate = chills, nausea, backache or fever.
** Poly = polyvalent vaccine with 400 CCA Asian and 75 CCA each of PR8, Swine, PR301 and B/GL.
Each test was with *80–97 college students per group* (16 to 30 years old)

to it and even 5 per cent of this group complained of moderate constitutional symptoms! Headaches and sore arms were reported by 13.7 per cent of these persons. The vaccines given intracutaneously, so that only 40 or 70 CCA were administered, produced essentially the same number of minor complaints as did saline, and even 200 CCA subcutaneously did not result in more reactions than in the placebo group. All of the 400 CCA lots produced more sore arms and headaches than did the saline, but the number of moderate reactions was not increased. It was of interest to find that the subjects who received the 700 CCA units per ml of polyvalent vaccine did not report more reactions than did the groups receiving 400 CCA units.

In view of the fact that influenza vaccines are prepared in embryonate eggs, an occasional severe reaction can be expected in egg-sensitive individuals. Sensitivity to eggs and the existence of an acute febrile illness are the only conditions which are generally considered contra-indications to vaccination The problem of vaccinating patients with histories of egg-sensitivity is being studied by at least one allergist (GAILLARD, 1957). Patients with positive skin tests for egg albumin have been injected with minimal quantities of commercial vaccine and sera have been obtained for antibody determinations.

C. *Evidence of Protection.*

The crucial test is, of course, whether vaccinated individuals are more resistant to clinical influenza than are unvaccinated persons. Two kinds of information relative to this matter are now available. The first was derived from artificially induced Asian influenza in vaccinated and unvaccinated volunteers. Secondly, there are already some preliminary observations from use of current vaccines under field conditions

An experiment conducted by BELL AND ASSOCIATES (1957) during the summer established that subcutaneous injection of 200 CCA vaccine did provide protection for a majority of subjects (ages 21 to 57 years) who were challenged two weeks later with throat washing materials from typical cases of Asian strain influenza. Although there were only fifty-five volunteers in the test and conclusions are therefore subject to the variation inherent in small numbers, the effectiveness of the vaccine was indicated as 46 per cent. Eighteen of the 23 who had not been vaccinated became ill while only 14 of the 32

vaccinated individuals developed disease. The correlation between HI antibody titers and immunity was evident. The attack rate among those who had not been vaccinated and were without antibody was 78 per cent. Those vaccinated who had not developed detectable antibody were infected at a 60 per cent rate. In the group with antibody levels of less than 40 the attack rate was 40 per cent, and of those with antibody titers of 40 or greater only 25 per cent became ill. In view of the fact that the material contained approximately 1,000 egg infectious doses per ml the intranasal instillation and throat swabbing with that amount of virus might constitute an unusually severe challenge as compared to that encountered in natural circumstances. The illnesses which developed in the non-vaccinated subjects were typical of those described during the 1957 pandemic. The impression was gained that the symptoms were usually milder in the vaccinated subjects and "were less likely to be disabling or of as long duration".

As director of the Commission on Influenza of the Armed Forces Epidemiological Board, DAVENPORT (1958) has released certain news about preliminary observations made at military stations where carefully controlled studies of influenza vaccines are in progress. Several different monovalent and polyvalent vaccines are being evaluated. With the exception of one lot prepared according to the formula of the 1956 polyvalent vaccine without Asian virus, all other test materials contain at least 200 CCA units per ml. Vaccines with 250, 400, or 750 CCA units per ml of an Asian strain have also been injected in a sufficient number of subjects at least ten days before occurrence of influenza in control groups to allow estimations of effectiveness. Although scheduled laboratory tests have not been completed to define the incidence of influenza in various groups, an approximation is possible on the basis of admissions to hospitals or reported cases of acute respiratory illnesses. With these criteria the rate has been approximately three cases in control groups to one in the subjects vaccinated with 200 CCA units. More precisely, estimates of the vaccine effectiveness range from 53 to 60 per cent. Attack rates in a representative study were 70.2 per 1000 per week in a placebo group and 28.4 per 1000 in those receiving vaccine. There was suggestive evidence that the effectiveness of vaccines at 400 or 750 CCA levels would be on the order of 60 to 75 per cent. The details and discussion of these experiments will appear in publications of completed studies.

Although much has been written concerning the monovalent Asian strain vaccine, hope of preventing influenza lies in the use of multi-strain or polyvalent vaccines. There is little room for argument as to whether the vaccines of this nature are of value. The question is, and has been for several years, how best to compound them, and by what immunization schedule should they be given to provide a maximal prophylaxis against influenza. It is becoming increasingly evident that durable immunity against each set of variants can be established by vaccination. It can be predicted that arrangement of the course and route of injections to obtain such a state will soon be thoroughly understood and applied. A far greater challenge, however, will be to compile facts concerning the antigenic structure of influenza viruses with a logic that will lead us to a promised land of stout, high, immunological walls which are impervious to, and cannot be scaled by, troops of antigenic aliens.

VIII. Conclusions

Asian strain influenza had a profound impact upon the world in 1957. Mass communication media made the most of an eminently newsworthy situation. The public was kept remarkably well-informed of the progress of the disease, of vaccine research, and vaccine production Indeed, persons concerned with influenza intelligence at times found themselves relying upon the newspaper and wire service dispatches for information about the latest events. Public interest in the new pandemic initially was centered around three items: was the pandemic to be like that of 1918 in terms of extent and mortality; was there going to be a vaccine available in time to meet the epidemic; and would this vaccine be of any value? Virologists and epidemiologists were interested in these questions, of course, but they were even more interested in the possibilities that the pandemic might provide for new advances in knowledge and for confirmation of virologic and epidemiologic concepts that had been long conceived but never established for want of a major natural epidemic situation.

After some months it became apparent that the influenza was as mild as the usual disease of the inter-pandemic epidemics of recent years. Complications were few and deaths even fewer, although mortality statistics were significantly affected. A vaccine was produced of moderate effectiveness in a number of countries, including the

vaccinated individuals developed disease. The correlation between HI antibody titers and immunity was evident. The attack rate among those who had not been vaccinated and were without antibody was 78 per cent. Those vaccinated who had not developed detectable antibody were infected at a 60 per cent rate. In the group with antibody levels of less than 40 the attack rate was 40 per cent, and of those with antibody titers of 40 or greater only 25 per cent became ill. In view of the fact that the material contained approximately 1,000 egg infectious doses per ml the intranasal instillation and throat swabbing with that amount of virus might constitute an unusually severe challenge as compared to that encountered in natural circumstances. The illnesses which developed in the non-vaccinated subjects were typical of those described during the 1957 pandemic. The impression was gained that the symptoms were usually milder in the vaccinated subjects and "were less likely to be disabling or of as long duration".

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many studies initiated this year can be reported. Only then will the true picture of the pandemic come into sharp focus, and the value of results derived from tremendous investments of funds, time, and effort expended by thousands of investigators can be assayed.

Postscript: Early in January the incidence of influenza began to increase again in the U.S. and many cases were seen during the next three months in localized epidemics. Furthermore, a marked elevation in mortality rates was seen in February and March which may have been influenza-associated. Industrial and school absenteeism rates were not increased during the early Spring experience, however.

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United States, the USSR, England, Germany, the Netherlands, Switzerland, Italy, the Union of South Africa, Japan, Australia, Canada, Brazil, Argentina, Denmark and Sweden. In a majority of these countries epidemics arrived before the vaccine programs had become fully established, but in a few, some vaccinations were carried out before the full impact of the epidemic was felt. The United States organized the largest scale program, and millions of doses were available before the epidemic started. In those countries with vaccine programs, priority systems were usually established. Initial supplies went to doctors, hospital personnel, essential employees in other fields vital to the community, and to old persons and the chronically ill. In general, it was possible to vaccinate many of those persons in priority groupings in the few countries with available vaccine. As a result of the great amount of activity in the vaccine field, a considerable body of new information is accumulating. The immunology of influenza is rapidly being enriched and ideal prophylactic measures are a realistic future prospect.

As the pandemic progressed, the fears about a repetition of 1918 subsided. During the fall a greater cause of general concern was the possibility of a second wave of epidemic disease in countries already once affected. In November this situation appeared to have occurred in Japan. Investigations reported at the time of this writing, however, suggest that in Japan a split epidemic situation was probably the explanation of the apparent second wave rather than a true reinfection second wave. By early December it seemed more than likely that the pandemic was generally nearing its end.* Most epidemiologists and virologists agreed, however, that the new Asian set would persist in the population in a sub-epidemic or local outbreak fashion for some years to come. Previous experience in this century suggests that the Asian variant might be dominant among type A viruses for as long as ten years.

From the point of view of the professional person in the field of public health the mobilization to meet the pandemic has been of inestimable value. An opportunity has been provided for unification and improvement of many public health facilites. Many laboratories, previously ill-equipped for, and with little skill in virus diagnostic work developed new facility before the end of the pandemic (JENSEN AND HOGAN, 1958). It will be several years before results of all the

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* See *Postscript* p. 205.

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TICK-BORNE SPRING-SUMMER ENCEPHALITIS

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Introduction

Tick-borne spring-summer encephalitis (synonyms: tick-borne encephalitis, Russian spring-summer encephalitis) is a seasonal virus neuro-infection transmitted to persons by wood ticks of the family Ixodidae. It was first detected (1935–1937) in the forest regions of the Far East (Far East Forest encephalitis), and later (1938–1940) in Sibieria and various regions of the European part of the USSR.

After 1948, it was described in many Central European countries (Czechoslovakia, Bulgaria, Yugoslavia, Austria, Hungary, Poland). The disease is endemic to the many forest regions where ticks of the *Ixodes persulcatus* or *Ix. ricinus* species are found.

The related disease entity (biphasic meningoencephalitis) may also be transmitted peroraly by milk from goats bitten by ticks in the forest pastures.

The clinical characteristics of the sickness were first studied in the Far East by PANOV, A.G. (1936, 1938).

The etiologic agent of the disease was discovered in 1937 (SILBER, L.A. and co-workers, 1938, 1939 a, b, c). Epidemiological aspects and the vectors of the infection were studied by the scientific expeditions in 1937–1939 under SILBER, L.A. (1938–1939), PAVLOVSKIY, E.N. (1938, 1939), SMORODINTSEV, A.A. (1938, 1939a, b, 1940, 1944), and CHUMAKOV, M.P. (1938–1940). In 1938–1939, a method was developed for producing active immunity by a vaccine prepared from formalinized, killed virus (SMORODINTSEV, A.A., KAGAN, N.V., and LEVKOVITCH, E.N.). In 1949–1953, SMORODINTSEV, A.A., and DAVIDENKOV, S.N. described, in addition to the classical paralytic form, a so-called biphasic meningo-encephalitis which is widely spread in the European part of the USSR. This type of tick-borne encephalitis, which runs a benign clinical course, is frequently transmitted by the drinking of infected goat's milk (SMORODINTSEV, A.A. and co-workers, 1949, 1953; DAVIDENKOV, S. N. and co-workers, 1925). A similar form of the disease is prevalent in Central Europe (BLASCOVIC, 1954; LIBIKOVA, 1954; RASKA, 1954; RÀMPAS AND GALLIA, 1949; VAN TONGEREN, 1955b; VERLINDE, 1956; KMET and co-workers, 1955; FORNOSI, 1954, OKER BLOM, 1956; GRINGSCHL, 1955; PRZESMYCKI, 1954a).

I. Biological and Antigenic Properties of the Virus

The viruses of the paralytic and double-phase type of disease are similar but not identical as regards their pathogenicity for various laboratory animals. They have an identical antigenic structure, regardless of the site from which they are isolated (patients, ticks, warm-blooded animals). The virus is closely related to the virus of loupingill encephalitis of sheep in Scotland.

The diameter of the virus is 20–25 millimicrons (CHUMAKOV, M.P., 1944). It passes, without much loss of its infectivity, through Berkfeld, Chamberland and Seitz filtres. It preserves its activity for over 3 years when kept in the cold (0–4° C) in a 10–20% brain suspension or in small pieces of infected brain held in a 50% glycerol. It is more stable when it is kept frozen at a temperature of −30 to −75° C, or after it has been dried by lyophilization. It is highly sensitive to the action of ultraviolet rays and is readily inactivated by heat. A 5% brain suspension was inactivated in 5–7 days at 37° C, in 60 minutes at 60° C, in 1–2 minutes at 100° C. The sensitivity to chemical agents (formalin, lysol, alcohol) depends on the type of tissue in which the

virus was grown. It is definitely lower in a 5% brain suspension than in the allantoic fluid of infected chick embryos. The virus as present in a 5% brain suspension of infected mice was inactivated in 0.2% formalin in 1–2 days at 37°, 8–10 days at 18° and 21 days at 2–4°C, (SMORODINTSEV, A.A., AND CO-WORKERS, 1941). The virus, inactivated by formalin, retains its immunogenic activity after lyophilization, even without prior removal of the formalin by dialysis or neutralization.

The virus multiplied preferentially in the brain of infected white *mice (log average titer 8.5)*, in EHRLICH's ascites carcinoma, and significantly less in chick embryos (6.5–7.0) and in monolayer cultures of the epithelium from monkey kidneys. The low potency of the formalin-killed vaccine obtained from the tissues of chick embryos compared to that of the vaccine from the brains of white mice is explained by the difference in the original concentration of the virus in the chick embryo and in the mouse brain, the difference being 1.5 to 2.0 $\log_{10}$.

The best way of infecting the developing chick embryo is in the yolk. The higher concentration of the virus is detected in the embryonic tissues (7.0) and in the yolk tissue (7.0), the lower in the allantoic and amniotic fluids (5.5–6.0), and in the allantoic membrane (SLONIM, 1956). The virus multiplies and maintains itself when passaged in *suspended or monolayer cultures of human embryonic tissue, monkey* kidney, chick embryo, and mice without regularly producing cytopathogenic effects. To propagate the virus under laboratory conditions, suspended or monolayer cultures of chick embryo are very useful. The cytopathogenic action of the virus in explants of the allantoic membrane of the chick embryo is not very regular. More constant changes are found in HeLa cultures, which are useful for titration of the virus and for neutralization tests. The test in HeLa cultures becomes complicated because of the early appearence of spontaneous degeneration of the cells. *The concentration of the virus* after 3–8 days of culture in mouse muscle tissue or in chick embryo lung tissue is 2.5–3.0; in human embryo muscle tissue and in HeLa cells 6.5–7.0 ($\log_{10}$). A gradually increasing cytopathogenic action was noted during 9 passages in cultures of the fibroblasts from explants of human embryo muscle. More intensive cytopathic changes were obtained with laboratory strains than with freshly isolated strains (LIBIKOVA, H., 1956; OKER-BLOM, N., 1956).

On fibroblasts from human embryo explants, the virus produces cytopathic changes *which are visible 24–48 hours after infecting the*

culture. The maximum titer of the virus is determined in 4–8 days. The cytopathogenic affect could be maintained for 9 consecutive passages, and could be depressed by specific anti-serum (ZASUHINA, G. D., AND LEVKOVITCH, E. N., 1957).

The antigenic structure of the virus strains of tick-borne encephalitis, which were isolated in various geographical regions of the Far East, Siberia, the Urals, in Western regions of the USSR, in Central Europe (Czechoslovakia, Austria, Yugoslavia), is very homogeneous and does not depend on the clinical and epidemiological characteristics of the illnesses with which they were associated (STRAUSS AND KOLMAN, 1954; PESEK, 1954, POND AND RUSS, 1955; BARDOS, 1956; VESENJAK-ZMIJANAC, 1955; GRINGSCHL, 1955). This is equally true of the parent virus, louping ill, which does not differ antigenically and immunogenically from tick-borne encephalitis virus.

Unexpected was the discovery that the etiologic agent of Omsk hemorrhagic fever had an antigenic structure closely related to the tick-borne encephalitis virus (CHUMAKOV, M. P., AND CO-WORKERS, 1954).

To study the antigenic relationships of the viruses indicated above we used: (1) complement fixation tests, (2) cross neutralization tests in white mice, (3) neutralization tests in developing chick embryos or in tissue culture, (4) cross neutralization tests with immune sera, exhausted by heterologous strains, (5) cross resistance tests in mice By testing the intracerebral resistance of cross-immunized mice CASALS (1944) differentiated the virus of tick-borne spring-summer encephalitis from that of Scotch encephalitis (louping ill). This could not be confirmed in our laboratory (FEDORCHUK, L V., 1954).

The viruses of the paralytic form (Eastern and Western variants) and of the biphasic form were found to be identical to that of louping ill of sheep The findings of some authors (SILBER, L. A , 1946; IVANENKO, A. I., 1953) as to the possibility of differentiating these viruses in cross neutralization tests in mice can be explained by the low precision of the method Considerable fluctuations in the results may be due to the varying individual resistance of different animals. Considerably greater precision could be obtained when the cross neutralization tests were carried out in chick embryos or in tissue culture, and the subsequent presence of free virus determined by passage to white mice, or by complement fixation.

II. Experimental Infection of Susceptible Animals

The viruses of the paralytic and biphasic form of tick-borne encephalitis are pathogenic for a wide variety of laboratory, domestic, and wild animals. The clinical picture of the experimental infection which most nearly resembles that in human beings occurs in monkeys (*Mac. rhesus*) infected by the intracerebral route (ILJENKO, V. I., AND POKROVSKAYA, O. A., 1958; PRZESMYCKI, F. ET AL., 1954 b).

The virus of the paralytic form of tick-borne encephalitis produces in monkeys a rapid development of flaccid paralyses of the upper and lower limbs starting in proximal parts and gradually passing to the distal. Epileptic-like fits with myoclonic, tonic and clonic convulsions are frequently present, fibrillar twitchings of the affected muscles and nystagmus are occasionally noted. Cerebellar ataxia is absent (S. also SILBER, L. A., AND CO-WORKERS, 1953; SHUBLADZE, A. K., and SILBER, L. A., 1945).

The virus of Scotch encephalitis (louping ill) produces a completely different clinical picture, the principal sign of which is the cerebellar ataxia leading to a complete inability to perform complicated movements, such as taking of food. The infected monkeys take food with difficulty or not at all, are incapable of holding on to the mesh of the cage, etc. An intensive tremor and a horizonto-rotary nystagmus of cerebellar type are developed. Epileptic-like convulsions are not often present and then only in a weak form (ILIENKO, V. I., AND POKROVSKAYA, O. A., 1957; GORDON ET AL., 1932; HURST, 1931). There are no paralyses of the limbs.

The virus of the biphasic meningo-encephalitis produces in monkeys clinical signs somewhat between those characteristic of the experimental Scotch and tick-borne encephalitis. More typical of this type of virus is the development of cerebellar atactic movements and intensive tremors, which is analogous to, although less severe than, that in Scotch encephalitis. On the other hand, in some animals, epileptiform convulsions and paralyses are observed, similar in their clinical course to those produced by the virus of the paralytic form of tick-borne encephalitis (increase of tendinous reflexes, etc.).

As can be observed from Table I, the clinical picture of the experimental infection in monkeys permits us to differentiate not only the viruses of the Scotch form, but also the viruses of the paralytic form from those of the biphasic form of the tick-borne encephalitis.

White mice are useful for the laboratory differentiation of the

Table I Principal Signs in Monkeys (*Macacus rhesus*) Infected Intracerebrally with Various Viruses of the Tick-borne Encephalitis Group

Virus Strains	Number of Infected monkeys	Paralyses	Epileptic convulsions	Increase in tendon reflexes	Cerebellar ataxia	Intention Tremor
"Sofin" (Paralytic form)	6	6	2	—	—	—
"Absettarov" (Biphasic form)	12	3	9	4	12	4
Scotch (Louping ill)	6	—	1	1	6	3

tick-borne and Scotch encephalitis viruses from the biologically related Japanese and Saint-Louis encephalitis viruses. The intracerebral inoculation of white mice with different encephalitis viruses produces a fairly uniform clinical picture of a severe paralytic process. However, subcutaneous or intraperitoneal infections are sharply differentiated by the highly pathogenic strains (under these conditions) of tick-borne encephalitis from the weaker viruses of Japanese or Saint-Louis encephalitis (OLITSKY AND CASALS, 1948; PRZESMYCKI, F. ET AL., 1954 a)

Three to ten days after mice have had a cerebral infection and 4–12 days after an intraperitoneal infection with the tick-borne encephalitic virus (differences in the length of incubation depend on the dose and the degree of virulence of the tested strain) they develop signs of excitement and of increased sensitivity to tactile, sound and pain irritations, and loss of equilibrium. The motor activity decreases, tonic and clonic spasms develop, which pass into paralyses of limbs and back. A few hours after the onset of the paralysis, the animals die. Strains of the paralytic form often cause paralysis of the hind legs, and strains of the biphasic form paralysis of the fore legs. Shortly before the mice die their skin and rectal temperature drops to 23° C.

In intraperitoneal infection of white mice with small doses of the paralytic or of the biphasic form a more rapid involvement of the brain is noted in the paralytic form than in the biphasic form. The virus of the biphasic form mutiplies most intensely in the first days after the infection, in the spleen, is found regularly in the blood, and it penetrates the central nervous system of infected mice somewhat later than the virus of the paralytic form (ILIENKO, V. I., 1956).

In studying the dynamics of virus dissemination in mice after infecting them subcutaneously or enterally, two principal phases in the development of the experimental infection can be observed:

(1) multiplication of the virus in reticulo-endothelium tissues (spleen) with a scarce and slow development of the virus in the brain;

(2) explosive multiplication of the virus in the brain, leading to a rapid process of sickness in the animals and to their death.

The amount of virus in the internal organs during this period decreases. Mice are most sensitive to the intracerebral infection, to a lesser degree to the intraperitoneal, intranasal, intravenous or subcutaneous inoculation of virus. The amount (LD_{50}) in 1 gm of brain tissue of infected white mice depends on the method of titration and is expressed by the index $\log_{10}$; intracerebral infection 8.0–8.5, intraperitoneal 6.5–7.0, intranasal 6.0, subcutaneous 4.0–5.5.

Virus strains of the biphasic form, freshly isolated from patients' blood, goats' milk and starved ticks, frequently have low indexes of activity in extraneural (such as intraperitoneal, subcutaneous) infection of white mice. However they rapidly increase after 8–10 additional passages of the strains through the brains of white mice or developing chick embryos (SMORODINTSEV, 1956).

White mice are susceptible to peroral infection with 10% suspension of infected brain (fed with food) and also to rectal infection; this produces 50–75% of takes. The high sensitivity of white mice to minimum doses of the tick-borne encephalitis virus was a determining factor in using them for the isolation of virus from the blood and C.S.F. of sick persons, from the brain of fatal cases, from vector ticks and from warm-blooded animals. The virus may be isolated from the brain in more than 80% of fatal cases, if the death occurs before 7 days from onset of the disease and if the brain tissue is removed as soon as possible after death. The virus may be isolated from patients' blood during the acute fever period, but yields no more than 30% positive results. Only rarely has virus been isolated from cerebrospinal fluid.

The virus from fatal cases is frequently isolated in the first direct passage in white mice. To isolate the virus from blood and cerebrospinal fluid, it is necessary to perform 2–3 supplementary blind passages in order to enrich the virus. Similar increases in concentration of virus may be achieved by inoculating these specimens into the allantoic cavity of 7–8 day old chick embryos. Four to 5 days after incubation, a passage of the yolk sack and allantoic membrane is made into the brains of healthy mice.

Guinea pigs are susceptible to intracerebral infection; the virus in adult animals multiplies poorly, producing a light infection with 2–3 days' rise in temperature. Infected newborn and young pigs develop pareses and paralyses of the limbs with fatal outcome As in white mice, the virus multiplies to high degree in the spleen and also in the mammary glands of females. Guinea pigs are more useful in obtaining high titer complement-fixing antiserra (after immunization with a live virus).

Analogous changes in adult susceptibility are observed in white rats, pigs, chickens and cats. Adults are fully resistant to the intracerebral infection, whereas newborn animals are highly sensitive to all paralytic and biphasic strains of the virus and also to that of Scotch encephalitis (DROBYSHEVSKAYA, A. I., ILJENKO, V. I., FEDORCHUK, L. V., 1954, PESEK, 1956, BARDOS, 1955). Adult and newborn syrian hamsters are susceptible to the tick-borne encephalitis virus. Cotton rats are not very susceptible to the intracerebral introduction of tick-borne and Scotch encephalitis viruses and illness is produced less regularly than in young white rats.

In studying the relationship between the viruses of tick-borne and of Scotch encephalitis (louping ill), great attention was paid to findings on sheep which are natural hosts to Scotch encephalitis virus. Intracerebral inoculation of sheep with tick-borne encephalitis virus produces fever (41–41.5°C), convulsions, prostration and death. According to the data of SILBER, L. A., AND SHUBLADZE, A. K. (1944) and ZAHAROVA, M. S. (1949), virus of the Far East tick-borne encephalitis, after a 5–6 day incubation period, produces in sheep paresis and paralysis of the limbs, and also vertigo with the sheep walking in large circles The tick-borne encephalitis virus, isolated from *Ixodes ricinus* ticks in Western regions of Byelorussia, produces ataxia with an unsteady walk and sharp leap-like movements in a circle. Towards the end of the sickness, paralyses and pareses of limbs develop In sheep the strain of Western tick-borne encephalitis produced 3 different types of disease. (1) vertigo, (2) paresis, (3) without any signs of nervous system involvement (ZAHAROVA, M S., 1949). In infected sheep the following principal syndromes of the disease are observed: (1) vestibularcerebellar, (2) comatose, (3) paretic, (4) subclinical (SILBER, L A., AND CO-WORKERS, 1949).

Identical symptoms were produced by the Scotch encephalitic virus The authors observed the presence of cross-cerebral immunity

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(1) Paralytic form of polio-encephalomyelitis A severe form occu in the Far East and Siberia. The Eastern spinal variant produces mortality rate of up to 25%. A mild form occurs in the Europe part of the USSR. The Western meningeal variant produces a mo tality rate of up to 5%. The clinical form of the disease was studi by: PANOV, A. G. (1936, 1938, 1951, 1956), SHAPOVAL, A N. (194 GLASUNOV, I. S., AND SHAPOVAL, A N. (1940), GRASHCHENKOV, N (1941, 1944), DAVIDENKOV, S. N. (1952), DAVIDENKOV, S. N. a co-workers (1952), HANZAL AND HENNER (1954).

An acute onset is characteristic of the paralytic form and a hi fever (39.5–40 5°) occurs 8–14 days after infection There is a rap development of meningeal symptoms such as neck rigidity, KERNIC and BRUDZINSKI's signs

Against a background of general tiredness, weakness, sleepine clouded consciousness, disorientation and delirium, some of t patients develop flaccid paralyses and a rapid atrophy of the muscl of the shoulder blade, the region above the shoulder blade, t deltoid muscle, the biceps and triceps (more pronounced in t proximal parts of upper limbs). After this there may be persiste paralyses of the neck and shoulder muscles, and of the upper lim (Figure 1)

When the patient is comatose, the bulbar centers are affected T patient has difficulty in swallowing, breathing, and moving l tongue Death may occur after 1–2 days of sickness, but more fi quently 4–7 days after onset. The percentage of mortality fluctuat between 5% and 30% It is higher in the Far East and significant lower in the European part of the USSR

In 5–8 days the temperature falls by lysis, the crisis drop is mo rare. Consciousness becomes clearer, and meningeal symptoms gr dually disappear. The recovery period is long, up to 1–2 months

As the diagnosis of tick-borne encephalitis has improved in t past few years, the percentage of mild cases has been increasing a the majority of cases show meningeal symptoms without intensi involvement of the central nervous system.

The clinical variations in the paralytic form of tick-borne enceph litis are as numerous as in poliomyelitis. The disease may take rapid, lightninglike bulbar form (in which a fatal outcome is ve frequent); a form of acute polioencephalomyelitis with flaccid pa lysis, a chronic progressive form with an epileptic syndrome KOJEVNIKOV type, or, finally, more benign or abortive meninge

between the viruses of the Western form and of the sheep vertigo but not between the viruses of the Eastern and Western forms of tick-borne encephalitis. The basic conclusion of SILBER, L. A. that the virus of tick-borne encephalitis, isolated in Western parts of the USSR from *Ixodes ricinus* ticks, is identical to that of sheep vertigo, remains open to dispute. The absence of disease among sheep in the most active regions of infection of tick-borne encephalitis in the Western parts of the USSR does not support this conclusion. An intensive (natural or experimental) contact of sheep with infected carriers in forest stations stimulates the formation of intensive humoral immunity, but does not produce vertigo. (FEDORCHUK, L. V., 1954; SMORODINTSEV, A. A., AND CO-WORKERS, 1954, 1955). At the same time, the clinical differences in experimental infections of sheep with the Western or Eastern variants, testify to a different pathogenicity of these viruses in sheep.

In the experimental peripheral infection of sheep with the paralytic and biphasic form of tick-borne encephalitis virus, an asymptomatic form of infection develops. The virus is frequently secreted with the milk, which may have epidemiological significance (KARPOV, S P., AND TIUSHNIAKOVA, M. K., 1958).

Goats are susceptible to the natural (tick-bite) and experimental infection of the paralytic and biphasic forms of tick-borne encephalitis viruses Intracerebral inoculation of the virus is followed by the rapid development of paresis and paralysis of the limbs terminating in death (LEVKOVITCH, E. N., KAGAN, N. V., 1941). Peripheral infection of adult goats results in very mild or in an asymptomatic infection, with the regular secretion of virus in the milk, infection of young goats results in paralysis and ataxia (SMORODINTSEV, A.A. and co-workers, 1954; ILIENKO, V.I., 1956; VAN TONGERN, 1955 a, 1956).

The same was observed when goats were infected peripherally with the viruses of Scotch encephalitis or of Omsk hemorrhagic fever (DROSDOV, S G., 1958). In contrast to the paralytic form, the virus of the biphasic form frequently produces a biphasic type of fever curve in experimentally infected goats.

Clinical Forms

Two principal clinical forms of tick-borne encephalitis are known. They are frequently encountered sporadically in various large territories of the USSR.

(1) Paralytic form of polio-encephalomyelitis. A severe form occurs in the Far East and Siberia. The Eastern spinal variant produces a mortality rate of up to 25%. A mild form occurs in the European part of the USSR. The Western meningeal variant produces a mortality rate of up to 5% The clinical form of the disease was studied by: PANOV, A. G. (1936, 1938, 1951, 1956), SHAPOVAL, A. N. (1949), GLASUNOV, I S., AND SHAPOVAL, A. N. (1940), GRASHCHENKOV, N. I (1941, 1944), DAVIDENKOV, S N. (1952), DAVIDENKOV, S. N. and co-workers (1952), HANZAL AND HENNER (1954).

An acute onset is characteristic of the paralytic form and a high fever (39.5–40 5°) occurs 8–14 days after infection There is a rapid development of meningeal symptoms such as neck rigidity, KERNIG's and BRUDZINSKI's signs

Against a background of general tiredness, weakness, sleepiness, clouded consciousness, disorientation and delirium, some of the patients develop flaccid paralyses and a rapid atrophy of the muscles of the shoulder blade, the region above the shoulder blade, the deltoid muscle, the biceps and triceps (more pronounced in the proximal parts of upper limbs). After this there may be persistent paralyses of the neck and shoulder muscles, and of the upper limbs (Figure 1).

When the patient is comatose, the bulbar centers are affected. The patient has difficulty in swallowing, breathing, and moving his tongue. Death may occur after 1–2 days of sickness, but more frequently 4–7 days after onset. The percentage of mortality fluctuates between 5% and 30%. It is higher in the Far East and significantly lower in the European part of the USSR

In 5–8 days the temperature falls by lysis, the crisis drop is more rare Consciousness becomes clearer, and meningeal symptoms gradually disappear. The recovery period is long, up to 1–2 months.

As the diagnosis of tick-borne encephalitis has improved in the past few years, the percentage of mild cases has been increasing and the majority of cases show meningeal symptoms without intensive involvement of the central nervous system.

The clinical variations in the paralytic form of tick-borne encephalitis are as numerous as in poliomyelitis The disease may take a rapid, lightninglike bulbar form (in which a fatal outcome is very frequent); a form of acute polioencephalomyelitis with flaccid paralysis, a chronic progressive form with an epileptic syndrome of KOJEVNIKOV type, or, finally, more benign or abortive meningeal

forms. However, the meningeal forms may be mild at the outset, but they frequently pass into a more severe polioencephalomyelitic form (STEBLOV, E. M., 1941; BELINKY, N. L., AND SHAPOVAL, A. N., 1949; BELMAN, E. L., 1948, 1950). This never occurs in biphasic meningoencephalitis.

(2) *Biphasic meningoencephalitis.* This is characterized by meningeal symptoms, mild irritability and irregular encephalitic symptoms, and biphasic temperature reaction. After a mild clinical course of the first wave of the disease, an afebrile period sets in, often simulating complete recovery, after which most patients develop a more severe second wave of disease. The outcome of the disease is always favorable, without death or permanent impairment of the central nervous system (DAVIDENKOV, S. N., AND CO-WORKERS, 1952, 1953, 1954; VISAN, E. M., AND CO-WORKERS, 1950; BEDJANIC, 1955; JUBA, 1955; GRINSCHGL, 1955).



a

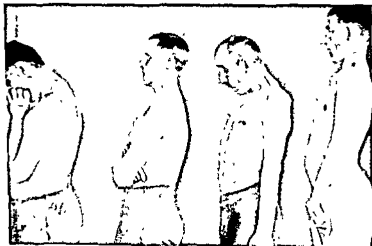


b

Fig. 1.
Paralyses of muscles of neck and shoulder blade after a paralytic form of tick-borne encephalitis (a–e)


c


d


e

In the first fever wave, the general symptoms are characteristic; they last for 2–8 days, and usually consist of shivering, headache, giddiness, nausea, and vomiting. *Meningeal symptoms are rare.* The face is hyperemic, the sclera are injected; there are pains in the muscles of back, neck, waist and limbs. Constipation is present; the tongue coated.

After the temperature falls, there is an afebrile period lasting 5–8 days. The patients feel fairly well, although most of them complain of weakness, general malaise, headache which is worse in people who stayed up during the first wave of the illness.

The second fever wave is more severe (Figure 2) The temperature rises for 2–3 days up to 39°–40° and remains elevated for 7–10 days. An unbearable headache frequently develops. The patients lie without moving, particularly avoiding any movements of the head. The face is hyperemic, the sclera injected. It is painful to move the eyeballs; *the patient has photophobia and is hypersensitive to sound and* tactile irritations. Psychic symptoms are: general deafness, decreased memory and decreased reaction to his surroundings, and occasionally, psycho-motor excitement. The majority of patients are extremely apathetic and without energy; their sleep may be affected (insomnia or drowsiness). The heart beat is dull, the pulse slow, the arterial pressure decreased. Some patients have an enlarged liver and spleen.

Against the background of general toxemia neurological symp-

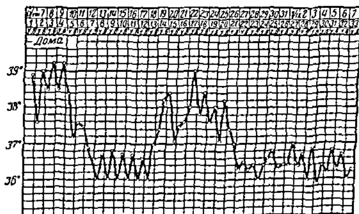


Fig. 2. Temperature curve of 3 5-year-old-child sick with biphasic meningoencephalitis. In the second phase, virus was isolated from the cerebrospinal fluid.

toms develop, pointing to the involvement of the cerebral membranes and the brain substance.

In the majority of patients meningeal symptoms, of moderate or mild degree, are found; sometimes they are expressed only in rigidity of the neck. The meningeal symptoms are usually transitory and rapidly disappear. Lumbar puncture reveals a moderate lymphocytosis (50–70 cells); when the spinal fluid is left standing a cloudy film frequently forms.

In a number of cases separate involvement of the peripheral nervous system occurs. There is pain along the nerve trunks when they are pressed or stretched, a decrease or loss of tendon reflexes, a lowering of muscle tonus, and a neuritis of optical, facial or auditory nerves.

In more than half of the cases, mild symptoms of involvement of the cerebral parenchyma are found; such as mild pyramidal hemisyndrome, reflexes of oral automatism, appearance of separate extrapyramidal symptoms (amimia, tremors) Frequently a more defined cerebellar syndrome develops, it can be more generalized (cerebellar walk) or more local such as asynergetic elementary movements. Sometimes there is a true intention tremor. Vertigo is sharply expressed; it frequently interferes with the sitting up in bed of the patient or it may cause an unsteady walk. In some cases, a thalamic syndrome is observed.

A number of cases had anisocoria, myosis, difficulty in convergence, optic sympathetic syndrome, and diplopia Numerous disruptions in the vegetative nervous system are observed; sharp disturbances in sweating (profuse hidrosis, occasionally dryness of the skin), lowering of blood pressure, sialorrhea, disturbances of pain adaptation, increased electro-cutaneous resistance, disturbances of thermoregulating reflexes, tendency to hypoglycemia, increased excitability of the peripheral nerves, and occasionally involvement of the sphincters.

Muscular spinal paralyses do not develop in the described forms except for occasional temporary neuritis or radiculoneuritis. In a few isolated cases these disturbances were of myelo-radiculitis In 15–25% of patients only one wave was noted, the degree and length of which usually corresponded to the second wave of the biphasic manifestation

In the clinical picture of illness, symptoms of the nervous system and very mild changes of the internal organs are most prominent The pulse is slow, the liver and the spleen enlarged, and are painful

In the first fever wave, the general symptoms are characteristic; they last for 2–8 days, and usually consist of shivering, headache, giddiness, nausea, and vomiting. Meningeal symptoms are rare. The face is hyperemic, the sclera are injected; there are pains in the muscles of back, neck, waist and limbs. Constipation is present; the tongue coated.

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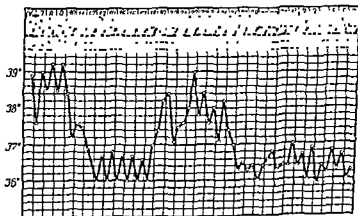


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during palpation; everything rapidly returns to normal. In some cases kidneys are affected; pyelitis or pyelo-nephritis may be present. Occasionally, a rapidly disappearing rash of erythematous character is present. In the first days of illness, there is constipation, the appetite is poor. Leucopenia is found, lymphocytosis in the first fever wave, and moderate leucocytosis with normal leucocytes during the second bout of fever. An increased monocytosis was not observed in the first or second bouts of fever. Erythrocyte sedimentation is usually increased and remains high for a long time. No changes in the urine are observed, if the kidneys are not affected.

The outcome of biphasic meningo-encephalitis, as a rule, is favorable. Not one of 2,500 patients whom we observed died from the original disease. The recovery from neurological disturbances was usually complete, and only the involvement of the vegetative nervous system persisted longer. After the illness, a prolonged state of inertia or general neurotic symptoms of neurasthenic type were observed fairly often; then conditions frequently require special treatment.

A chronic course of the disease was not observed in any of the cases.

There were no essential differences in the clinical course of the disease, whether caused by tick-bites or by drinking infected milk. The clinical picture of the disease in different geographical areas was identical.

The clinical course of the biphasic form is so typical and standard that physicians familiar with this illness usually have no difficulty in giving the right diagnosis on the basis of the clinical data. Typical epidemiological properties of the disease are: the season, relationship to a tick-bite or the drinking of raw goat's milk. The characteristic clinical course distinguished it from a number of similar illnesses (leptospirosis, influenza, listerellosis).

The biphasic disease differs from the classic type of tick-borne encephalitis (Far Eastern and Western variants), by the absence of atrophic paralyses, including those of the neck muscles. The bulbar syndrome and the fatal outcome are not observed, nor is there progressive involvement, in particular KOJEVNIKOV epilepsy. In the classic paralytic form of tick-borne encephalitis, no connection with drinking infected goat's milk was found.

Epidemiology

The basic epidemiological pattern of tick-borne encephalitis as a transmissible infection is a reflection of its natural history (PAVLOVSKIY, E. N., 1944) In the light of PAVLOVSKIY's, E N. study on the natural history of the disease, the virus, which is transmitted by bloodsucking Ixodes ticks, is maintained in nature by the process of its uninterrupted circulation between the vector and the wild and domestic warm-blooded animals which provide nourishment to the blood-sucking vectors. The movement of tick-borne encephalitis virus from one host to another is presumed because of the presence of certain fauna and of favorable climatological factors

Characteristic of the natural history of tick-borne encephalitis is the correlation between the virus, its donor, its vector, and its recipient which arose in the process of evolution without any dependence on man, who is in most cases an accidental link in the circulation of the virus. Ecological, biological, and physiological characteristics of the vector are most important in the epidemiology of tick-borne encephalitis, as it is in any other transmissible disease (PAVLOVSKIY, E. N., 1944).

The vectors of this disease—wood ticks of the family *Ixodidae*, *Ixodes persulcatus* and *Ixodes ricinus*—are important and permanent reservoirs of the virus of tick-borne encephalitis in nature Isolated species such as parasitic mites, infesting rodents' holes, and ticks *Haemaphysalis concinna* and *Dermacentor silvarum*, may also circulate the virus, but their role as vectors remains to be determined (Fig 3)

The biological cycle in the wood tick's development and the dissemination of the virus are inseparably tied to the warm-blooded animals on which the vectors feed Adult ticks feed on cows, goats, sheep, horses, elks, wolves and hares Larvae suck blood from wood mice, shrews, field mice, and moles. Nymphs feed on hares, chipmunks (*Eutamias sibiricus* Lax), squirrels, and field mice. Birds play an important role in transporting infected ticks over great distances, thus encouraging the introduction of new hosts for tick-borne encephalitis. Wild birds which are the most frequent carriers of ticks are: thrush (*Turdus pilaris* L.), nutcracker bird (*Nucifraga caryocatactes* L.), tree pipit (*Anthus trivialis*), hammer-bird. (*Emberiza citrinella* L.) and others (SOLOVIEV, V. D., 1939, 1941; MOSKVIN, I. A., 1940; SMORODINTSEV, A. A. and co-workers, 1939; DROBYSHEVSKAYA, A. J., AND NEUSTROEV, V D., 1943.)

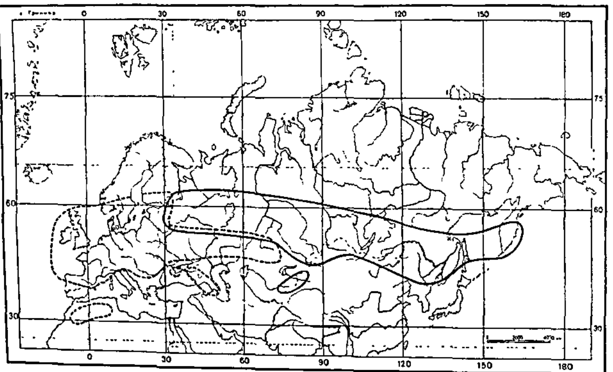


Fig 3. Geographical distribution of ticks *Ixodes ricinus* (dotted line) and *Ixodes persulcatus* (solid line)

Frequent additional carriers of ticks can be: hamsters, *mice*, field mice, squirrels, *forest mice*, hares; and among the birds, jay bird, bullfinch, chaffinch In a number of other animals larvae and nymphs are found only rarely (nutcracker bird, field mice, mole, harvest mice, titmouse, ermine, water mice, magpie, weasel, and bank swallow).

Although the number of hosts upon which the tick vectors feed is very great and includes more than 100 different species of mammals and birds, the chief factor in maintaining the infection in any area depends upon the prevailing animals and birds. A periodic increase in the number of ticks in Czechoslovakia follows upon a year of sharp increase in the mouse population ("mice years" from HANZAL, F., 1954).

Tick-borne encephalitis is endemic in wide forest areas (occupying about 50% of the USSR territory). It includes Siberian forests, mixed transitional forests of the European part and the Far East, mixed and broad-leaved forests along river valleys, stream valleys and forest regions in the steppes. Mixed and broad-leaved forests, in horizontal and vertical belts, are the most dangerous for tick-borne encephalitis (Fig. 4).

Attempts were made to classify the natural hosts of tick-borne encephalitis according to climatic, and geographical and botanical characteristics of various forest areas. The specific biological characteristics of the host determine the principal ways in which the virus circulates in inter-epidemic and epidemic seasons.

These characteristics essentially influence the length of the epidemic season, which fluctuates in the Far East, depending on the microclimate, from 2 to 4 months At present, endemic areas are found, not only in the Far East and Siberia, but also in various forest regions of the middle and northern belts of the European part of the USSR (CHUMAKOV, M. P. and co-workers, 1939, 1940, 1943, 1944).

Tick-borne spring-summer encephalitis is a very old disease breeding in various forest regions of the USSR and beyond the frontier. Cases of tick-borne encephalitis, at the end of the last century and the beginning of this century, were found in the Far East and the Urals (SMORODINTSEV, A. A., 1939a, 1939b, DANKOVSKIY, N. L., 1939).

The spreading of the disease, for the most part, in thick forest regions, far removed from qualified medical institutions, explains the complete unfamiliarity of physicians with it before 1935 (PANOV, A. G., 1936)

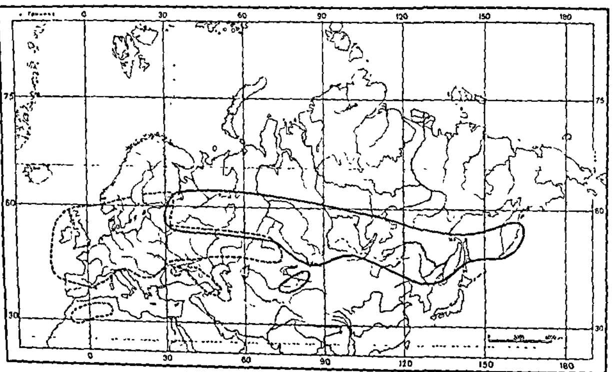


Fig 3 Geographical distribution of ticks *Ixodes ricinus* (dotted line) and *Ixodes persulcatus* (solid line).



Fig 7 Tick *Ixodes ricinus*, nymph 50 ×

During this time they increase in size up to 1 5 mm and they drop off the host the underbrush Within the next 40–100 days the larvae turn into starving nymp with 4 pairs of legs (Fig 7) The nymphs hibernate and in the spring attack the warm-blooded hosts (mice, rodents, chipmunks, rabbits, squirrels) and suck blo for 5–6 days The satiated nymph, lead-gray in color and up to 3 mm in si changes in the depth of underbrush, by autumn or the following spring, into t adult male or female tick

The length of the entire cycle, from the egg stage to an adult tick, is 3 yea during this time the tick changes hosts three times (POMERANTSEV, B P, AND SE DLKOVA, G.V, 1939, 1948) May and June is the period when the greatest number ticks are found in the adult stage and when they are the most aggressive (active their attachment to man and animal), after this period the ticks in this sta disappear

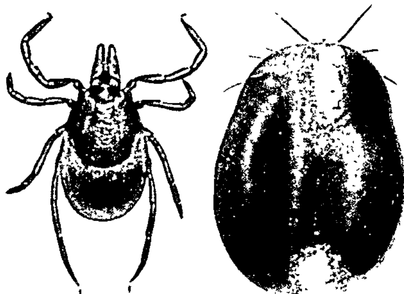
Larvae and nymphs are found during spring and summer months

Ticks can become infected with virus only through wild animals, but n through man The tick attached to a man's body always leaves after sucking t blood for a few days If a satiated tick drops in a building it will perish, becau it is not able to realize its metamorphosis because of the lack of the necessa natural conditions

The climatic conditions of a geographic region determine the beginning of t season in tick activity In most areas of tick-borne encephalitis in the USSR it the middle of April, when the 24-hourly mean temperature of the air is 3 to 4° The ticks' attack on man occurs at a 24-hourly mean temperature 10 to 12° C, wh corresponds to the end of April or beginning of May.

The activity of ticks decreases at a temperature above 18°

The absolute number of encephalitis vectors in forests is n always in proportion to the number of illnesses The latter is dete mined by the percentage of virus-infected ticks and the extent



5. Tick *Ixodes ricinus*, hungry female 12 × 6 Tick *Ixodes ricinus*, satiated female, 6 ×

male tick there is the thorax, dark brown in color, of hard chitin, it occupies about half of the female's lenght Behind the thorax there is a softer chitin, reddish-orange in color, it stretches in sucking In males all the back is covered with dark, hard chitin, it does not stretch in sucking (Fig 5)

The fine sense of smell in ticks helps them to concentrate in the paths where animals and people walk Ticks climb on stems and grass leaves, but not higher than 0 5 meter from the ground Waiting for many days for a passing animal, they spread to the side their front pair of legs with which they quickly attach themselves to the animals 'fur or to the peoples' outer clothing They attack day and night, on sunny or rainy days, hiding in the ground only during heavy rains, but there is a remarkable periodicity in tick activity during a single day

The male feeds only for a few minutes and sucks in only a little blood The female sucks blood without interruption, up to 9 days, at this time it is fecundated by the male.

The satiated female considerably increases in size (up to 10–12 mm) and transforms into a blood-filled egg-shaped sac of lead-gray color (Fig 6). The satiated female leaves her host independently and in 10–12 days lays 600 to 3,000 eggs in forest ground or in soil cracks; after that she dies

In the beginning of August, from the eggs tiny (less than 0 5 mm) pale yellow, starving larvae develop, they resemble in shape the adult ticks, but have only 3 pairs of legs

The starving larvae hibernate and in the spring attach themselves to small vertebrates (mice family or insectivores) to fill themselves with blood for 3–5 days

munity to the tick-borne encephalitis virus which develops as a result of subclinical infection following repeated contacts with ticks. The more intensive the circulation of virus in the vectors and the more frequent their contact with persons, the higher is the number of patients and the percentage of persons immune through exposure to infected tick-bites. Consequently a gradual decrease of sick cases occurs amongst the old inhabitants of a given region. However, when a new susceptible population moves into an area where the disease is not overt, a large number of cases may occur.

The most important habitat of ticks is the forest. The most favorable habitats are mixed fir and leaf forests along the lowlands, with growth of linden, older trees, and shrubwood Ticks are found more rarely beyond forests and shrubwood. Ticks hibernate in all their stages of development, except ova and hungry larvae. Hungry larvae perish at –7°C and lower.

The role of pasture ticks, *Ixodes persulcatus* and *Ixodes ricinus*, as vectors is proved by the frequent isolation of encephalitis virus when a suspension of ground starving ticks (gathered in nature) is injected into the brains of white mice. This virus was found to be identical to the virus isolated from sick people (MIRONOV, V. S., 1938; SHUBLADZE, A. K., AND SERDIUKOVA, G. V., 1939; PAVLOSKIY, E. N. and co-workers, 1947; RAMPAS AND GALLIA, 1949; SMORODINTSEV, A A. and co-workers, 1954).

The most sensitive and effective method of isolating the virus from ticks found in nature is by intracerebral injection of 4–6 white mice with a suspension of culture larvae, bred in the laboratory from individual females, taken from the woods in a hungry state, or from cattle in a satiated state. Every 5–7 days, 2–3 passages are done on the brains harvested from infected mice.

In many regions of the European part of the USSR, the isolation of virus from hungry adult female ticks, which were tested in groups of 20, was almost nil (one virus strain per 50–100 groups or per 1–2 thousand ticks) Similar groups in the larval state laid by individual females (1,000–2,000 larvae from each culture inoculated into a group of mice) usually produce 5–25% positive takes for virus This can be related to the large number of larval samples tested and to the large concentration of virus in them (CHUMAKOV, M.P., 1944; SMORODINTSEV, A.A. AND CO-WORKERS, 1953; GULAMOVA, V.P. AND OLENEV, N.O., 1954).

Infected wood *Ixodes* ticks transmit virus to susceptible animals by biting

which the population comes in contact with these ticks. Outbreaks of tick-borne encephalitis have been described in regions with low numbers of ticks not heavily infected with encephalitis virus, where there was an intensive contact by a considerable group of people with the natural hosts (MELNIKOV and co-workers, 1949). Many of the known areas of tick-borne encephalitis are well inhabited by man and are in the direct neighborhood of towns and heavily populated areas. Rare cases of infection occurring in homes have also been described, through ticks brought into the house on bouquets of forest flowers, on grass, or on hunter's clothing.

The role of ticks in the infection of human beings is easily confirmed in most patients by epidemiological investigation.

In the usual case history, there are definite indications of bites and of the removal of sucking ticks 8–15 days before the onset of the disease. The patients had been in the woods and exposed to ticks.

The curve of encephalitis infections corresponds to the population curve of the ticks in nature. The time of their appearance, their maximal development, and the ceasing of their activity somewhat precede the curve of infection. As the adult pasture ticks disappear from nature, so does the illness decrease among people.

The quantitative distribution of encephalitis cases is closely correlated to the territorial distribution of ticks: illnesses are more numerous in fir and leaf mixed forests, rich in tick fauna, and rare in such forests as pine, birch and oak, where there are not many ticks or none at all. In the population exposed for a long period to repeated tick-bites, a gradual dying out of encephalitis is observed, which is related to the development of immunity. Always a small number of people bitten by infected ticks become ill, and most of these persons become immunized.

After a bout of the disease, virus-neutralizing antibodies remain in the blood for many years, usually for the whole life span; complement-fixing antibodies disappear in 1 to 2 years. Persons who have had the disease do not get it a second time, as they possess a lasting immunity.

In endemic areas, there is always a great number of permanent inhabitants who have not had clinical tick-borne encephalitis, but who have neutralizing antibodies. The percentage of such persons is proportional to the length of their stay in the endemic regions and to the intensity of their contact with ticks. Often more than 50% of the adult population in an endemic region may have humoral im-

their warm-blooded hosts and infect them. From these animals healthy larvae, nymphs and imago are infected which in turn transmit the virus to the next stages in development and also transovarially. In order to infect a tick a high concentration of viremia is important (SARMANOVA, E. S., 1948; PATTYN AND WYLER, 1955).

Each new type of animal, including man, which enters the tiaga regions teeming with virus reservoirs of ticks and rodents, is subjected to tick attacks and to encephalitis infections.

From this it follows that spring-summer encephalitis is not primarily a disease of human beings but exists in nature independently of man. The attack of ticks on man is an incidental episode, since people do not play a role in the normal life-cycle of ticks. This role belongs to various wild and domestic animals.

In the biphasic form of tick-borne encephalitis, the infection occurs not only through direct bite of the tick but also through the alimentary tract in drinking milk from goats bitten by ticks in pastures (SHTILBANCE, 1952, SMORODINTSEV, A. A. and co-workers, 1953). The alimentary portal of entry of biphasic virus meningoencephalitis has been proved by direct isolation of virus strains from the milk of goats naturally infected in the pasture in two different areas of infection (SMORODINTSEV, A. A., ILIENKO, V. I., AND CO-WORKERS, 1953). In various regions one way of infection may prevail over another, depending upon the number of goats owned by the population and the intensity of their contact with wood-ticks. In one of the areas which we studied, up to 70% of all illnesses were produced through the use of infected goat's milk. These illnesses bear the character of a family-group outbreak; they occur in the same period in the course of several days amongst a group of persons drinking milk from the same goat. In other areas of tick-borne encephalitis not more than 5–20% of illnesses are related to the alimentary infection via goat's milk; the majority of cases are caused through direct contact of patients with ticks. These cases of infections through ticks are of sporadic character and they seldom produce other cases in the family (SMORODINTSEV, A. A. and co-workers, 1953, 1954; RASKA, K., ET AL., 1954; ILIENKO, V. I., 1956).

In this connection it is not correct to name the biphasic form of tick-borne encephalitis "milk biphasic fever" (CHUMAKOV, M. P., AND CO-WORKERS, 1954). This name designates only one epidemiological variant of the disease (alimentary infection via milk) and ignores the no less frequent sporadic illnesses transmitted through tick-bites.

Ixodes persulcatus and *Ixodes ricinus* ticks free of virus are easily infected when fed on mice, guinea pigs, and rabbits infected with encephalitis virus. The ingested virus multiplies intensely in the body of the tick and is easily transmitted to other animals through biting (RIYJOV, N. V., AND CO-WORKERS, 1940).

The ability of infected ticks to transmit the virus to susceptible animals through biting is explained by its distribution in the tick. The virus concentrates essentially in the salivary glands of ticks (PAVLOVSKIY, E. N., 1939) and also in the intestinal tract (SMORODINTSEV, A. A., AND ILIENKO, V. I., unpublished).

Ticks act as the basic reservoir of virus by their ability to transmit the agent transovarially. This has been followed through several generations (PAVLOVSKIY, E. N., 1944; CHUMAKOV, M. P., AND CO-WORKERS, 1945), and is of important epidemiological significance.

In the opinion of PANOV, A. G., (1956) and others, the duration of ovarial transmission of the virus by *Ixodes* ticks depends on the conditions of their feeding. If the blood-sucking occurs on animals, without specific virus neutralizing antibodies in their blood, transmission of the virus from one stage of development to another goes unchecked. If the blood sucking occurs on animals immune to tick-borne encephalitis, transmission of the virus to the next stage of development is impeded. But this conclusion is not sufficiently confirmed by experimental data.

The ticks *Ixodes persulcatus* and *Ixodes ricinus* infect man, and they are regularly encountered in all endemic areas of tick-borne encephalitis. They outnumber other types, and they are frequently infected spontaneously with the encephalitis virus and easily infect *susceptible animals through biting.*

Domestic and wild animals, particularly rodents, are an additional and closely related reservoir of the encephalitis virus in nature. In contrast to the protracted circulation of the virus in the tick, the virus is preserved in the organism of warm-blooded (susceptible and non-susceptible) animals for a very short time, not over 1–2 weeks.

This is confirmed by finding the virus for short periods in the brain and blood of wild animals trapped in encephalitis areas (chipmunk, field mice, hedge hog, mole, rabbit) and also in various birds (grouse, yellow hammer, thrush). Various rodents inhabiting forests are also susceptible to the virus.

A description of the virus cycle in the encephalitic area follows: larvae, nymphs and imago naturally infected with the virus attack

incorrectly thought to be cow's milk, when in fact it was mixed with goat's milk.

Results of a study on the alimentary outbreak of tick-borne encephalitis in Rojniava (Czechoslovakia) is of a great interest. The outbreak started in the end of April 1951, the illnesses were difficult to diagnose and were thought to be influenza. After May 5, more than 660 persons fell ill, half of them diagnosed as cases of the single phase form of the disease; the other half clearly had the biphasic character. Family-group illnesses were observed with, however, no cases of contact infection The characteristic symptoms of the biphasic illness were drowsiness and coated tongue. Lumbar punctures relieved severe symptoms. The cerebrospinal fluid did not show a rise in protein or in cells, at any stage of illness or recovery. The outcome of the disease was favorable without permanent damage. The only death (a 60 year-old man) was due to another cause: in the deceased person's brain histopathological changes of encephalitis were found.

The large outbreak which developed was related to an alimentary infection via raw milk (BLASCOVIC, RASKA, 1954). The principal source of infection was the local dairy which did not pasteurize cows' or goats' milk, but mixed the incoming produce for retail sale. It was established that most patients drank raw milk taken from this dairy, although some of them received milk directly from the animals

On the basis of laboratory and clinical research it was proved that neither bacteria, nor rickettsiae, nor leptospira were etiologically involved.

Serological examinations established the presence of antibodies to the viruses of Czechoslovakia, and Russian spring-summer encephalitis.

In 1952 zoological and parasitological research was carried out in the Rojneava region. Considerable titers of antibodies were discovered in various wild animals (deer, etc.), domestic animals (goats, cow, etc.) and in small mammals. The virus strains isolated from persons and ticks were identical to the virus of the Russian tick-borne encephalitis (ROSICKY AND HAVLIK, 1954; HAVLIK AND KOMAN, 1957; MACICKA, 1956).

Correlation Between the Biphasic Form of Tick-Borne Encephalitis and Scotch Encephalitis (Louping Ill)

In the opinion of SILBER, L. A., (1946), the milder cases of tick-borne encephalitis in the Western regions of the European part of the

Sharp differences in the incubation period and in the epidemiological characteristics of the biphasic form of tick-borne encephalitis are observed depending on the source of infection. The disease caused by tick-bites has a sporadic character; it develops in 8–20 days after contact, affecting mostly adult men engaged in physical labor in forest regions. The epidemiological link in this case is the transmission of the virus from tick to man.

The alimentary diseases have a family-group character and affect rapidly one person after the other in the majority of persons drinking milk from the same goat 4–7 days before the onset. Many in this group did not have contact with the forest nor did they remove any ticks from their body 8–20 days before the onset. The epidemiological link in this case is the transmission of the virus from tick to goat to man.

In this family group of biphasic meningoencephalitis there are many children of pre-school age and old people having no contact with the forest. The occupational group sharply differs from the group infected via tick-bite (SMORODINTSEV, A. A. and co-workers, 1953, 1954, 1955).

Cases of alimentary infection are frequently observed amongst the town population close to natural habitats of biphasic meningoencephalitis. Town inhabitants may become ill after spending Sundays in the village where they may have drunk goats' milk but did not walk in the woods nor did they find ticks on their bodies. In the same village, at the same time, other persons drinking milk from the same goat became ill.

Group outbreaks in towns have been related to the introduction of the virus with goats' milk delivered from country areas to the patient's house or bought at the market (see later, outbreak in Rojniava—Czechoslovakia).

The illness in goats produced mild signs of general inertness and a decrease in milk yield.

The virus of biphasic meningoencephalitis after subcutaneous inoculation into laboratory animals regularly finds its way into the milk of susceptible animals (goats, sheep, white mice, guinea pigs) but is not found in the milk of non-susceptible animals (white rats, rabbits) (ILIENKO, V. I., 1956).

No epidemics have been traced to infected cow's milk; this becomes understandable when the resistance of cows to the virus is taken into consideration. Occasionally the source in infectionof persons was

The prophylaxis of tick-borne encephalitis is carried out as follows:

1. *Control of vector ticks.* This includes measures directed to protect the population from the sucking and biting of ticks; also measures to destroy ticks in their native habitat. For protection against ticks, it is recommended to wear correct clothing in the forests which will impede the ticks from penetrating to the body; also the use of special protective preparations is recommended, such as phthalates, 10% carbonic soap emulsion, tar mixtures These preparations should be used to saturate belts, shorts, ties or arm bands (cloth or gauze) for they repel ticks.

Persons working in forests should be examined for ticks repeatedly (lunch hour and in the evening) to remove and destroy ticks immediately (PERVOMAYSKIY, G. S., 1940, ESKIN, V. A. and co-workers, 1944)

To destroy ticks on plants, on the ground, or on warm-blooded animals, a number of measures are taken:

(1) Economic measures. Improvement in organizing the cutting of forests rich in ticks during autumn—winter period; limiting the pasturing in forest stations of domestic cattle, on which the adult ticks feed

(2) Destruction of ticks on plants and in grass. To fight tick-borne encephalitis in the USSR the spraying of infested large forest territories with DDT preparations or with hexachlor (by ground or aviation dusting) has proved to be successful. A 10% dust of DDT or hexachlor in the amounts of 30–50 kg per hectare is used. According to the observations of GORCHAKOVSKAYA, N. N and co-workers, (1953, 1958) the destruction of adult ticks (*Ixodes persulcatus*) two years after a DDT spraying was 99 6–99 7% effective. The population of young ticks, determined by the number of larvae and nymphs on rodents decreased in one year of spraying of large forests by 97 6%, the second season it decreased by 96.6% and the third 99.5%. A greater effectiveness was observed in a small section of forest, where 4 years after the spraying no young or adult ticks are found

In spraying the forest with hexachlor (30 kg per hectare) adult ticks one year were destroyed up to 99 9%; however, in the 2nd and 3rd season ticks were found again, to a considerable number (30%). The number of larvae and nymphs in various areas decreased unevenly (20–84%). This can be explained by the less stable and shorter action of hexachlor than that of DDT. The latter remains during several

USSR (for instance Byelorussia), transmitted by ticks *Ixodes ricinus*, are etiologically independent and are caused by the virus of Scotch encephalitis. According to the data of SILBER, L. A., AND ZAHAROVA, M. A., from these patients and from ticks *Ixodes ricinus* (taken from infection areas) strains of Scotch encephalitis were isolated which differ antigenically from the virus of tick-borne encephalitis (SILBER, L. A., AND CO-WORKERS, 1944, 1945, 1946).

The sera of persons who have had this disease, neutralize chiefly the Scotch encephalitis virus and to a lesser degree the tick-borne encephalitis virus. These laboratory data were not confirmed in repeat tests. The sera of persons having had encephalitis in Western areas neutralize equally all known varieties of tick-borne encephalitis (Eastern and Western variants of the paralytic form and the virus of the biphasic form) and the virus of louping ill. The virus strains isolated in Byelorussia from the tick (*Ixodes ricinus*) do not differ antigenically from any other strains of tick-borne encephalitis and louping ill.

In Scotland and Northern England the epizootic type of encephalomyelitis amongst sheep, caused by louping ill virus, has been known for the past 150 years. However illnesses amongst persons living in these areas are very rare and data not reliable. On the contrary, in the Western forest regions of the USSR, where the vector of the Scotch encephalitis is spread—the tick *Ixodes ricinus*—many persons become ill every season but the vertigo of sheep is absent. We studied this problem in the regions where biphasic tick-borne encephalitis is found in Northwest and the near Urals regions where sheep breeding is intensive and where animals are allowed to pasture in forest regions teeming with infected vectors, *Ixodes ricinus* or *Ixodes persulcatus*. In spite of the intimate contact of infected ticks on young susceptible sheep and the development in the latter of a strong humoral immunity towards the end of autumn, no vertigo cases were found in herds up to 50,000 head of sheep. Therefore, the presence of Scotch encephalitis in the USSR is not proved.

The Scotch encephalitis affects only sheep, and is not dangerous to people; on the contrary, the tick-borne encephalitis is not at all dangerous to sheep under natural conditions while many persons fall ill. Although the clinical course of Scotch encephalitis in people infected in the laboratory is very similar to the biphasic form of tick-borne encephalitis, the two illnesses have completely different etiology.

The inoculation of the population in endemic areas radically changes the epidemiological situation, and when it is applied on a large scale to the menaced population, it can extinguish the disease even in the most dangerous areas of the Far East.

Lately, production of the dry preparation of tick vaccine has been started. It is very stable for long periods and during shipping (DROBYSHEVSKAYA, A. I., AND SMORODINTSEV, A. A., 1955; LEVKOVITCH, E. N., 1956).

The re-vaccination (with 3 ml of vaccine) should be done every 2–3 years. The possibility is being studied of producing a killed virus vaccine from virus grown in chick embryos (SHUBLADZE, A. K.). However, this vaccine produces a less defined immunogenic effect than the vaccine from the brain of mice. Attempts to obtain attenuated variants of the virus to create a live avirulent vaccine did not produce good results. This is due to the high stability of the pathogenic capacity found in the tick-borne encephalitis virus group.

The virus strains of biphasic meningoencephalitis have a very favorable outlook, as these represent natural strains of reduced virulence. They do not cause disturbances in movements or death of persons. Cases of laboratory infections caused by the biphasic encephalitis virus were not severe.

As regards unvaccinated persons bitten by ticks, seroprophylaxis is necessary; this can be a subcutaneous injection of 5–20 ml of serum or gamma globulin from hyperimmunized horses and goats, or from persons recovered from the disease. The seroprophylaxis decreases the number of illnesses amongst persons bitten by ticks, at least 4 and more times (GLAZUNOV, I. S., and co-workers, 1939; CHUMAKOV, M. P., 1940, ANDJAPARIDZE, O. G., ET AL., 1957; BARDOS V., 1956).

To produce the hyperimmune serum, horses are immunized subcutaneously with a 10% suspension of the brains of infected mice or chick embryos (120–150 ml of antigen for one course of 5 immunizations). The antigen is injected at 7–8 days intervals; 12 days after the final immunization, blood is drawn. After an interval of one month to maintain the titer of the serum, a new course of immunization is begun and so on up to 10 times.

seasons on the soil of forest underbrush. The spraying of DDT dust can be done early in spring or late in fall.

The *treatment of natural habitats of tick-borne encephalitis* with DDT produces a lasting freedom from ticks in forest regions, for the basic vectors perish.

Other blood suckers of rodents (mites, fleas, lice) are not affected or only slightly so. The study of a possible role of other blood-sucking vectors in transmitting the virus in areas freed of ticks *Ixodidae* by spraying, would be of a great interest. If other vectors exist besides the *Ixodes* ticks, they would assert themselves even if the principal vectors are completely suppressed.

(3) Extermination of rodents leads to the loss of the basic majority of young ticks (larvae and nymphs).

2. *Control of the alimentary infection by the milk of goats in forest pastures.* During the infectious season it is recommended to drink boiled goats' milk, and to improve the taste, it is recommended to mix it with raw cows' milk which is free from virus.

3. *Vaccination by the standard killed vaccine.* The specific immunity arising regularly in persons who had the tick-borne encephalitis can be reproduced for a limited time (1–2 years) by a subcutaneous introduction of our killed vaccine (5% suspension of infected mouse brain inactivated for 20 days by formalin, 1:600, at 0–4° C) (SMORODINTSEV, A. A. and co-workers, 1940, 1941; LEVKOVITCH, E. N., AND SMORODINTSEV, A. A., 1940).

White mice, vaccinated 3 times intraperitoneally with 0.25 ml of this preparation, are able to withstand up to 100,000 lethal doses of virus *when infected intraperitoneally, but they do not develop* an immunity to intracerebral infection. Two subcutaneous introductions of 3 and 5 ml formol vaccine are given to the people at an interval of 7–10 days. The epidemiological effectiveness of the vaccine was first proved in 1939. Amongst 925 woodcutters, who came to the Far East for the first time and who were vaccinated with a standard dose, there were two mild cases of encephalitis, without paralytic complications. In a control group of 1,185 unvaccinated persons, similar in composition, there were 27 cases, for the most part severe, ending in 7 deaths.

In 1940 amongst 9,578 persons inoculated with standard doses of 5% formol vaccine, there was one case of encephalitis and amongst 6,180 unvaccinated persons there were 38 cases with 9 deaths.

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RUSSIAN SPRING-SUMMER VIRUS IN INDIA

Kyasanur Forest Disease

By TELFORD H. WORK*

Human disease caused by any of the tick-borne Russian Spring-Summer (RSS) complex viruses was unknown in the tropics until the discovery and description of Kyasanur Forest Disease in Shimoga District of Mysore State, India, in March-April 1957 (WORK AND TRAPIDO, 1957; WORK ET AL, 1957). The clinical illness in man in India displays many features of Omsk Hemorrhagic Fever (BILIBIN, 1950; GAJDUSEK, 1953), now known to be caused by a virus of the RSS complex (MACLEOD ET AL, 1956; KONOWALCHUK, 1957), with no overt signs of encephalitis. Further evidence is thus provided that this group of viruses not only has an extensive geographical distribution but also marked variation in pathogenicity and tissue tropism.

Human RSS virus infection in India was first suspected in 1952 when neutralizing antibodies to Russian Spring-Summer Encephalitis virus (*RSSE*) *were demonstrated in 6 of 26 sera collected from* young adults at Kutiyana, a rural town near the Arabian Sea coast of the Saurashtra peninsula, now included in Bombay State in Western India (SMITHBURN ET AL, 1954; KERR AND GATNE, 1954). (Figure 1). The appearance of a significant number of RSSE positive

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Fig 1 Localities of RSS virus infection in India.

human sera in only one of 38 widely scattered localities of South India suggested the possibility of recent introduction of the virus into that region (SMITHBURN ET AL, 1954). The further significance of this finding in Saurashtra in relation to the recent outbreak of Kyasanur Forest Disease in Mysore, is at present under investigation.

An outstanding feature in the epidemiology of Kyasanur Forest Disease is the involvement of two species of wild monkeys, the langur, *Presbytis entellus*, and bonnet macaque, *Macaca radiata*. It was a report received on March 23, 1957 (RAO, 1957) of wild monkeys of these species dying in a forested area of Shimoga District that first attracted the attention of the Virus Research Centre. Since fatal epizootics in forest monkeys, such as have occurred in South and Central America, have heretofore been indicative only of sylvan yellow fever (TAYLOR, 1951; TRAPIDO AND GALINDO, 1956), it was suspected at first that yellow fever had invaded a continent where there is no evidence that it has ever existed. With this fatal epizootic in India, monkeys are incriminated in a second arthropod-borne virus

infection of serious consequence to man, although one in which ticks appear to be the vectors rather than mosquitoes.

On arrival at Shimoga on March 26, 1957, the investigating team learned from the District Health Officer that cases of a prolonged, prostrating, febrile, and often fatal illness had occurred in human inhabitants of villages adjacent to forest areas where dead monkeys had been observed (SHAMA RAO, 1957). The symptoms, signs and course of the disease simulated enteric fever, but results of WIDAL and WEIL FELIX serological examination had been essentially negative (NARASIMHA MURTHY, to be published). These cases had been reported February to April 1956 and January to March 1957, during periods when monkey deaths in adjacent forests were also reported.

It was on March 27, during investigation of these human cases (PRASANNA IYENGAR, 1957) at the Ulavi Primary Health Centre that the first freshly dead langur (black-faced) monkey, collected moribund in Kyasanur Forest, was brought for post-mortem examination. The first Kyasanur Forest Disease (KFD) virus strains of the RSS virus complex were isolated from this monkey (WORK AND TRAPIDO, *1957*, in press; BHATT ET AL, in preparation). On each of the subsequent two days, bonnet macaque (red-faced) monkeys found dead in the forest—one at Shigga and another in Kyasanur Forest—were autopsied and virus was isolated from organs of each. After post-mortem examination of the monkey at Shigga village the first acute and convalescent patients were examined. They were suffering from what was later to be called Kyasanur Forest Disease, named after the locality where the virus was first isolated from monkeys and later from human patients exposed there.

Clinical Features of Kyasanur Forest Disease

The salient features of what has become the classical history, symptomatology and clinical course were first observed in these Shigga villagers. They termed their affliction "the monkey disease" because of their observation that seeing or smelling a dead monkey in the forest was often a precursor to onset of this particular illness which they recognized as new.

A more detailed description of the clinical manifestations in seven cases has already been published as a preliminary report (WORK ET AL, 1957). Kyasanur Forest Disease is suspected in patients giving a

history of forest exposure within eight days of sudden onset of headache, fever, low back and limb pains and severe prostration, often associated with inflammation of the eyes, diarrhoea and vomiting by the third or fourth day of illness. Bleeding from the nose, gums, stomach and intestines may begin as early as the third day. The majority of cases, however, run a full course without overt hemorrhagic signs.

Physical examination during the first few days of illness shows an acutely ill, listless, and often prostrate patient, too sick to answer questions quickly or directly. Temperature may exceed 39.4C., but seldom rises higher. In some patients the pulse may be slower than would be expected from the temperature

Almost always there is inflammation of the scleral and palpebral conjunctivae, with occasional photophobia. There may be enlarged and soft or sometimes shotty cervical lymph glands. Soft axillary lymph nodes 1 to 2 cm. in diameter are common. Epitrochlear nodes are palpable occasionally. The neck is often stiff owing to guarding of painful spinal and cervical muscles. A papulo-vesicular eruption on the soft palate is frequent and when seen is a characteristic diagnostic sign. Rarely blood may ooze from the gums; epistaxis, sometimes noted only by dried blood about the nares, may occur; and gastrointestinal bleeding may be evidenced by hematemesis, melena or red blood in the stools. Such bleeding may continue for many days after fever ceases.

Some patients have a persistent cough which may produce blood-tinged sputum or even substantial quantities of blood. The lungs are usually clear by percussion and auscultation, except in patients producing blood-tinged sputum or frank red blood In these patients rhonchi, wet rales and crepitations may be heard. They are signs of serious lung involvement which may be a precursor to pneumonia.

The heart size and sounds are not abnormal In some cases there has been a bradycardia, though in most the pulse reflects the patient's temperature. There is no abdominal pain except in cases with massive gastro-intestinal bleeding The liver is not palpable, but the tip of the spleen may be felt, most often in those patients with generalized lymphadenopathy. Kidneys are not palpable.

No skin eruptions or discoloration have been observed and none of the patients gave a history or showed clear evidence of tick bites. However, there were occasionally reddish papules or fading macules in the axillary region which appeared similar to the lesions observed on

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the skin after manual detachment of nymphal ticks. After several days of illness the skin is dry, an indication of the marked dehydration in these patients.

In all cases studied there have been no physical signs of any specific neurological lesions. Cranial nerves remain intact. Superficial, deep and extremity reflexes are present and equal bilaterally. No paralysis or weakness was demonstrable by neurological examination.

Mental confusion, drowsiness and, rarely, transient disorientation have been seen. Considering the severity of illness, the toxic condition, the organic damage described under pathology, the high and prolonged fever, the nutritional deficit, the dehydration and the general prostration, it is difficult to attribute any of these mental signs to specific damage to the central nervous system by the virus. These patients recover their mental alertness and orientation completely, with subsidence of fever, unless hemorrhagic complications result in fatal termination.

Because of limited laboratory facilities, haematological studies in the 1957 epidemic were confined to erythrocyte, leucocyte and WBC differential counts and examination of urine and cerebrospinal fluid. Red cell counts were not significantly altered but there was invariably a marked leucopenia during the acute disease. White cells diminished to between 2000 and 3500 per cubic millimeter of blood. The leucopenia persisted until after the patient became afebrile, at which time a mild leucocytosis often ensued. Rarely was there any change in the differential count. Platelets were counted in the blood of one patient and found to be markedly diminished in number.

Albumin appears in the urine in most cases on the third or fourth day and lasts until the initial fever subsides. Granular casts and pus cells may also be found in the urine at this time but no erythrocytes have been seen.

Cerebrospinal fluid has persistently been clear, with no increase in cells or alteration in proteins, chloride or sugar. Virus has been isolated from spinal fluid in four of eight attempts.

The course of the disease is variable. The febrile period ranges from five days to two weeks. Temperature may be normal in the morning and rise to an afternoon peak. Vomiting, diarrhoea, headache, and limb and back pains as well as conjunctival inflammation may persist throughout this period but subside when fever ceases, leaving the patient listless and exhausted. One to two weeks after termination of the first febrile period fever may recur and last for one to seven days.

The fever, vomiting and diarrhoea produce marked dehydration in a few days, in part because village patients refrain from taking fluid by mouth in the belief that this treatment will stop the diarrhoea. The consequences of hemorrhage depend upon where it occurs and the amount of blood lost. If into internal organs such as the lungs, the prognosis is grave. If externally in small amounts, it contributes to the dehydration and if it continues for long, may result in shock.

Whatever the course, recovery is slow and patients with the best in supportive treatment may require a month or more to convalesce. During this period any physical effort is difficult and often results in tremors due to weakness of the muscles.

Since the disease is caused by a virus for which no known specific drug is available, treatment is supportive, to alleviate symptoms of pain and vomiting, to combat the dehydration, and to replace electrolyte and blood loss. Vitamin K and iron as well as whole blood transfusion and plasma are indicated in patients with hemorrhagic complications.

Pathological Findings

What we know of the pathology of Kyasanur Forest Disease has been learned from post-mortem examination of organs and tissues from three human cases that terminated fatally on the seventh, eighth and ninth days of disease, and from ten monkeys found moribund or at varying periods of time after death in forests of the epizootic area (IYER, LAXMANA RAO ET AL, to be published; IYER, WORK ET AL, to be published). Two of the human autopsies were done within two hours after death and the third within twelve hours.

Human pathology: Gross findings in two of the three cases were hemorrhage and consolidation in the lungs and massive hemorrhage into the gastro-intestinal tract. This hemorrhage was not attributable to any focal lesion but rather to a generalized oozing of blood into alveolar spaces of the lung and into the lumen of stomach, jejunum, ileum and colon where it was found in all stages of digestion, with frank red blood in the sigmoid.

The brain and meninges were hyperemic in all, but no other gross lesions were observed. The liver, spleen and kidneys were not grossly abnormal but may have been relatively enlarged.

The histological findings in the three human cases were essentially similar.

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Monkey pathology: Condition of the monkey tissues at time of fixation varied from fresh or well-preserved to obviously putrefactive. The pattern of pathological changes in the viscera was generally the same as that seen in human organs.

Liver. In the organs that were preserved, the normal architecture was in general retained with varying lack of uniformity of staining of the liver cells Focal necroses were generally more pronounced than in the human livers, and in general, polymorphs were the predominant cell of reaction Granular yellowish brown pigment was seen in a number of the livers examined In addition, distinct rounded eosinophilic bodies in the cytoplasm were sometimes seen The significance of these has not yet been determined In one instance several of the central hepatic venous radicles were occluded by thrombosis and infarcts of varying ages were seen in another. As in the human livers, there was more than a moderate prominence of the reticulo-endothelial cells, some of which appeared hypertrophic or even multinucleated. Variable numbers of inflammatory cells were seen in the portal radicles

Kidney As in the human kidney, the main alterations appeared to be in the lower part of the nephron However, the changes in the cortical convoluted tubules appeared to be more severe and extensive. Focal inflammatory infiltrates were seen rarely These changes were seen in both the poorly preserved and fresh specimens In a few kidneys there were seepages of recently exuded erythrocytes in and around the tubules of the medulla

Lung Changes were similar to but of less constancy than those seen in the human lung sections These changes included recent exudation of erythrocytes and plasma into the alveoli and prominence of the septal capillaries in some specimens.

Heart Small focal infiltrates of mononuclear cells were seen in a number of monkey hearts Histiocytes were observed in the ventricular myocardium. These changes are evidence of focal necrosis

The other visceral organs, including spleens and sections of the gastro-intestinal tract, showed no changes other than those described in the human cases

Brain Histological changes of a disseminated non-suppurative focal necrosis were clearly evident in two of the monkey brains In contrast to the absence of definite histological evidence of encephalitis in the three human brains, these two monkey brains showed unquestionable non-suppurative encephalitis It is noteworthy that in these two monkeys KFD virus was isolated only from the brain of one and brain and spleen in the other and not from any other tissue or from blood serum This suggests the possibility that KFD virus can become neurotropic in man.

In one other brain there was apparent evidence of recent premortal brain damage, but without overt features of encephalitis. The other brains revealed histo-architecture consistent with their stage of preservation, prominence of blood vessels, occasional petichiae and prominence of satellite glia There were no inclusions of any sort in any of the brains examined

Comment In both the human and monkey tissues the histopathological changes appear to be mild in comparison with the severity of the clinical illness. The parenchymatous organs such as the liver and kidney appear to have sustained the main pathological alterations.

Liver: There was a general maintenance of lobular architecture and occasional loosening or separation of the liver cords without any prominent congestion of the sinusoids. The liver cells were generally well stained, but in all sections there was patchy or rarely zonal variability of cytoplasmic staining. The nuclei were vesicular with few to a number of binucleated cells seen. Granular brownish yellow pigment was observed in the cytoplasm of liver cells in all three cases. In one case there also appeared distended biliary canaliculi, probably an indication of bile stasis.

There were rare focal necroses and moderate to marked prominence of the Kupfer cells, which occasionally appeared hypertrophic or multinucleated and presented evidence of erythrocyto-phagocytosis. There was moderate prominence of the Glisson's capsule with variable numbers of mononuclear inflammatory cells in the portal radicles. Sections of one liver showed inflammatory infiltration of the vascular walls and in another there was rare evidence of coagulation necrosis of the cytoplasm with extrusion of coagulated bodies in the sinusoids.

Kidney: The capsule was normal and there was distinct corticomedullary differentiation. Some of the glomeruli appeared congested and swollen and filled Bowman's capsule. Degenerative alterations of the cortical convoluted tubules varied in degree. These were characterized by loss of cytoplasmic outlines, dilatation of the tubules, fragmentation of the tubular cytoplasm and sloughing of the cellular debris into the tubular lumina. Nuclei of such tubules were either well preserved, pyknotic or rarely extruded. The basement membrane of these tubules was usually intact. Sections in one case showed small foci of interstitial groups of mononuclear cells and pigment-laden macrophages.

The changes in the medulla were less extensive and included deep eosinophilic staining and nuclear pyknosis of the cells of the collecting tubules. There was extrusion of these cells from the basement membrane into the lumen. Recently exuded blood was seen in some of the medullary tubules in one case.

Lung: There was a moderate pleuritis in one case. In all three the pulmonary alveoli revealed patchy consolidation with prominence of the septal capillaries and a predominantly hemorrhagic exudate in the alveolar lumina. This exudate, along with desquamated epithelium, was seen in the bronchiolar lumina. There was patchy inflammatory infiltration of the bronchiolar walls.

Spleen: In all, the Malphigian follicles were generally indistinct and the red pulp was prominent by comparison. There was a moderate to marked prominence of the splenic sinusoids and of the littoral cells, some of which were undergoing mitosis. In one case there was evidence of erythrocyto-phagocytosis.

There were no constant significant changes in the rest of the abdominal and thoracic viscera, including several sections of the gastro-intestinal tract.

Brain: Microscopic examination of numerous sections of the brain from all representative areas in all three cases disclosed general *preservation of normal* histo-architecture and of the constituent nerve cells. In all there was a variable degree of glial cell satellitosis in the sections of the cerebral cortex. In one case there were small perivascular seepages of erythrocytes without any reaction in the [illegible]

[illegible]

[illegible]

of the three cases.

complished by intracerebral inoculation of blood serum and suspensions of heart muscle, skeletal muscle, lung, liver, spleen, kidney and brain into infant (two-day-old) and three-week-old white mice. In most of the initial passages the infant mice began to sicken after an incubation period of three days and most were dead by the fourth day after inoculation The sub-adult mice exhibited an incubation period of 4–6 days and after a more prolonged prodrome, with ruffling of the fur and lassitude, developed a characteristic spastic hind limb paralysis. The mouse brain suspension titre from the earliest passages exceeded 10^{-8} to 10^{-9} with less than a log drop in titre after lyophilization. This behavior in mice has remained consistent through twenty-eight mouse brain passages.

The virus also infected sub-adult mice by the subcutaneous and intraperitoneal routes of inoculation but produced a lower brain titre which exceeded 10^{-6} only after a prolonged incubation period of 6–13 days.

In monkeys. Rhesus, *Macaca mulatta*, monkeys were inoculated, four IC and two IP, with the infected langur monkey sera. Only one monkey of each category circulated virus. The IC inoculated monkey circulated virus from at least the 4th through the 7th day and the IP on the 6th and 7th post-inoculation days. Sera collected from these two monkeys three weeks after inoculation neutralized more than three logs of KFD virus.

Experimental infection was produced in three macaque monkeys by injection of mouse brain KFD virus by the IC, IP and SC routes, respectively The viremia in each lasted at least seven days and all developed substantial amounts of specific neutralizing antibody. Overt disease has not been reproduced in rhesus or bonnet macaque monkeys but further experiments on this aspect are under way.

One hundred LD_{50} mouse neutralization tests on sera collected from monkeys in the infected area indicate that many survive the natural infection and some may sustain inapparent infections. The sera from five of twenty-five langur and one of twelve bonnet macaque monkeys collected within the known infected area contained neutralizing antibodies to KFD virus, while 13 monkey sera collected just outside the area were negative.

In man. The first two strains from human patients were isolated at the VRC field laboratory in Vellore from blood serum collected on the first and fifth days of illness. No monkey material or other strains of

The details of these changes are not referable to any particular disease process. They could be interpreted as consistent with toxemia incidental to virus circulating in the blood.

The hemorrhagic phenomena which were seen in some cases of the clinical disease have not been adequately explained by the histological appearance of the organs examined. The absence of recognizable alterations in the gross and microscopic anatomy of either the blood vessels or the tissues where these hemorrhages have occurred suggests that the defect in the hemostatic mechanism lies elsewhere. Histological changes indicative of a suppression of maturation of the cellular elements of the bone marrow have been described in some of the hemorrhagic fevers that have been encountered in the Soviet Union (GAJDUSEK, 1953). Kyasanur Forest Disease appears to be very similar to Omsk Hemorrhagic Fever (CHUMAKOV, 1918).

Putrefactive alterations in some of the monkey organs interfered somewhat with interpretation of the pathological changes observed in these organs. However, the changes described were qualitatively similar in the poorly and well-preserved specimens. Further evidence of specificity of these changes awaits interpretation of results of experimental monkey infections now under way in the laboratory.

Coupled with minimal evidence of CNS damage, the general pathological picture substantiates the clinical impression that Kyasanur Forest Disease is not primarily neurological. KFD also differs from the hemorrhagic fevers (GAJDUSEK, 1956; Symposium, 1954) caused by RSS and unrelated viruses in that there is less kidney involvement. These factors suggest that KFD is usually a mild disease which should ordinarily result in a favorable outcome if adequate supportive therapy is initiated early enough in the course of infection.

Dehydration has already been mentioned as a possible contributory cause of more severe clinical disease. This, along with other factors such as general state of nutrition, chronic disease and environmental adaptation, may contribute to a different course and pathological picture than would be observed in human beings infected elsewhere.

Nature of the Virus and the Host Response

In mice: As previously mentioned, the first Kyasanur Forest Disease viruses were isolated from a langur, *Presbytis entellus*, and two bonnet macaque, *Macaca radiata*, monkeys. These isolations were ac-

before any information was available on the nature of the disease present or its etiological agent. The sera were inoculated only after the onset of the illness.

In contrast to the high percentage of virus isolations from human blood sera, CSF has yielded the agent in only three out of eight attempts, on the first, fourth and twice on the seventh day of disease. Since virus was easily isolated from serum collected at the same time, the virus in the CSF may be a reflection of the viremia rather than of invasion of the CNS.

Throughout the Kyasanur Forest Disease investigations in 1957, the haemagglutination inhibition test (CASALS AND BROWN, 1954) has been used for arbor virus group identification, diagnosis and epidemiological studies. It was first used to determine that human convalescents had suffered an infection with some group B arbor virus (WORK AND TRAPIDO, in press) Since the area in which Kyasanur Forest Disease has occurred is otherwise relatively free of other group B arbor virus infections known to exist in India, the HI test has been used as a diagnostic tool in demonstrating rise in titre of HI antibodies in serial serum specimens from single patients. In sera from four and possibly five patients, it is clear that detectable HI antibodies specific for KFD virus appeared before CF antibodies, sometimes several days earlier. In one instance a second serum specimen collected on the 6th day of disease contained virus when the only group B HI antibody found to be present was for KFD virus in low titre. No CF antibody was detectable in this serum.

Extensive survey collections were made in villages in and around the area where human cases were occurring, to determine which areas had evidence of infection. The HI test was useful in screening these sera before determining by CF test whether KFD virus was specifically responsible for the infection.

Sera from more than 1200 persons obtained in survey collections in localities of the infected and adjacent areas were tested by HI and CF against Japanese B encephalitis, Egypt 101 West Nile, Trinidad 1751 type II dengue, and KFD virus antigens. Of 123 sera which were positive by HI against one or more of these antigens, 51 contained complement fixing antibodies only to KFD virus. An additional 14 had CF antibodies to both KFD and one or more of the other antigens. Thirty-two sera had HI and CF antibody spectra predominant for the Japanese B-West Nile complex and in these the CF antibody to KFD was minimal or absent.

this virus had previously been handled in this laboratory. The behavior of these strains in mice was similar to that described above for the monkey viruses. They were identified as being similar to or identical with the monkey strains by cross-neutralization, complement fixation (CF) and by haemagglutination inhibition (HI) tests using human convalescent sera against the monkey viruses and the immune monkey serum against the human virus antigens.

Sera collected from acutely ill and convalescent patients suffering from Kyasanur Forest Disease in March, April, May and June 1957, were all sent, refrigerated on wet ice, by rail more than 500 miles to the VRC laboratory in Poona for virus isolation and serological testing. Figure 2 shows the results of the isolation attempts. It is obvious that there is a prolonged viremia of at least ten days from the onset of the disease. That 45 acute sera collected from 36 patients during the first two weeks of disease yielded 35 strains after such transport and delay in inoculation, indicates that the virus is quite durable in refrigerated human serum and that the circulating virus is of sufficient titre to survive storage and travel. It also stresses the value of virus isolation as the most expeditious method for laboratory diagnosis of acute febrile cases, it being possible to isolate the virus in infant mice and to identify it by complement fixation test in 4 to 7 days after collection of the acute febrile serum specimen.

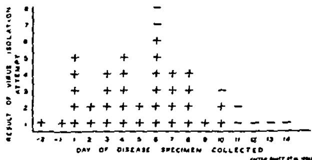


Fig 2. Isolations of virus from blood serum of 36 Kyasanur Forest Disease patients

The isolations from patients one and two days before onset of the disease, as shown in the chart (Figure 2), were from sera collected routinely for reference purposes from two VRC insect collectors (WORK ET AL, 1957). They had been exposed in Kyasanur Forest

RSSE virus infections. HI, CF and neutralization tests at the VRC soon showed that the Indian strains were closely related to RSSE virus (KULKARNI ET AL, in preparation).

Definitive identification of the monkey and human strains was carried out by Drs. CLARKE AND CASALS (1957) of the New York laboratories after they confirmed the VRC findings that the human patients had suffered from group B arbor virus infection and that the virus isolated from blood of persons suffering from the disease was the same as that isolated from the dead monkeys in the forest.

There was no perceptible difference between the monkey and human virus HI antigens prepared by the protamine-treated borate saline method. Both produced good haemagglutination at either room temperature or 37° C. over a pH range of 6.0 to 6 6.

Crude saline antigens for the two viruses were CF tested against pools of groups A and B arbor virus hyperimmune sera. No fixation was obtained with the A pool, while the group B mixture produced a positive result. Next a complement fixation test was set up using the P9605 human and the monkey virus antigens against the immune sera to thirteen other group B viruses and the Indian convalescent sera. Antigens to all thirteen group B viruses were tested against the convalescent sera.

The convalescent sera reacted strongly with the two new KFD antigens and also with RSSE antigen Hyperimmune RSSE mouse serum fixed complement to high titre with the two Indian KFD virus strains and RSSE antigen Serum and antigen dilution box CF titrations confirmed that the human P9605 isolate was similar to RSSE virus.

In rodents and small mammals Serum from the first langur monkey was inoculated IC and IP into different litters of suckling hamsters and cotton rats. The hamsters sickened and died but the cotton rats remained well. Suspensions of monkey brain, liver, spleen and heart muscle were inoculated IP into adult hamsters and guinea pigs without consequent sign of disease. These adult hamsters and guinea pigs, and the sub-adult cotton rats who had been inoculated as sucklings, were exsanguinated for serum to test for specific KFD virus antibodies. Complement fixing and haemagglutination inhibiting antibodies for KFD virus were present in the hamster and cotton rat sera, an indication that these animals had sustained a non-fatal and probably sub-clinical infection. The guinea pig sera showed no immune response.

Like other members of the RSS complex, KFD virus has proved to be highly infectious to human laboratory workers. Since initiation of these investigations, clinically apparent infections have occurred in nine VRC laboratory personnel, two New York Rockefeller Foundation Laboratories staff (CLARKE, 1957), and three members of the group producing vaccine at Walter Reed Institute for Medical Research in Washington (BUESCHER, 1957). None of the infections was fatal and the only one that caused serious concern was in an Indian member of the VRC staff, a laboratory worker at the Vellore field station who had not received prior immunization to any group B arbor virus. The other eight infections were in VRC personnel who had all received 17D vaccination. Three of these had had two inoculations of potent formalinized mouse brain RSSE vaccine, but in spite of the two RSSE vaccinations no specific neutralizing, CF or HI antibodies to KFD virus were detected in their reference sera. The disease in these three persons was a mild febrile illness of two or three days' duration. Virus was isolated from their blood serum and subsequently a marked antibody rise occurred.

The benign course of the infections in persons with prior antigenic experience with group B viruses, the manifestations of visceral rather than central nervous system involvement in overt Kyasanur Forest Disease, and the infection's apparent amenability to supportive treatment, suggest that the KFD member of the RSS virus complex may be one of the safer of these viruses to handle in the laboratory even though the infection rate has been high.

Identification of the Virus

Because of the epizootic involvement of forest monkeys and the presence of a visceral disease in man, attention was directed initially toward the possibility that the etiological agent might be similar to yellow fever virus. Neutralization and complement fixation tests of KFD virus strains against yellow fever immune reference sera as well as 17D yellow fever virus neutralization tests with convalescent KFD human sera, showed that although it was a III group B arbor virus, it was not closely related to yellow fever.

Lyophilized stocks of hyperimmune monkey sera to both yellow fever and Russian Spring Summer Encephalitis (RSSE) viruses were sent by air from the New York Virus Laboratories of The Rockefeller Foundation, whose staff had suggested that KFD might be due to

RSSE virus infections. HI, CF and neutralization tests at the VRC soon showed that the Indian strains were closely related to RSSE virus (KULKARNI ET AL, in preparation).

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The results of extensive trapping of small mammals in the forests of the Kyasanur Forest Disease infected and adjacent areas will be detailed in relation to the epidemiology. (Table II). Here, however, it is pertinent to mention that the three-striped palm squirrel, *Funambulus t. tristriatus*, and forest shrew, *Suncus murinus*, collected in the infected area gave significant incidence of neutralizing antibodies as compared to essentially negative results in the same species trapped outside the area. A lower incidence of antibodies was demonstrated in *Rattus rattus wroughtoni*, one of three species of rats caught in large numbers in the affected forests. This lower incidence of antibodies is considered significant because of the large number of sera tested from the species.

These results indicate that small mammals are infected with KFD virus and do produce specific neutralizing antibodies. The demonstration of inapparent infection in adult hamsters and cotton rats and the fact that no dead rodents have been discovered in the area, suggest the possibility that the virus behaves in a similar fashion in the wild small mammals. So far there has not been time for laboratory experiments with the small mammals or with avian species, although we have evidence of neutralizing antibodies in the jungle fowl, *Gallus sonnerati*, and the goldenbacked woodpecker, *Brachypternus benghalensis*.

In tissue culture: One of the most substantial achievements in the laboratory investigations of KFD strains of the RSS virus complex has been the primary isolation and adaptation of human and monkey virus strains in tissue culture (BHATT, in preparation).

Serum collected from a langur monkey which died in Kyasanur Forest was inoculated into monkey kidney (MK), hamster kidney (HK), guinea pig kidney (GPK) and chick embryo (CE) cells. No changes were noted in the monkey or guinea pig kidney cells and the harvest fluids did not affect mice. However, questionable cytopathogenic changes were noticed in the chick embryo cells on the fifth post-inoculation day and fluid harvested at that time produced death in all mice inoculated. Fluid harvested from the second CE cell tissue culture passage infected mice. Cytopathogenic changes in the cells were minimal but observable in the 11th CE cell passage, with harvest fluid virus pathogenic for mice.

The same monkey serum inoculated into hamster kidney cells showed questionable cytopathic changes on the third day. Third and twelfth day harvest fluid produced infection in mice and clear cytopathogenic changes were observed in second HK cell tissue culture passage.

The standard human KFD virus strain, P9605, has been adapted to both monkey kidney and hamster kidney cells with good yield of virus. The tissue culture LD_{50} titre at the 35th passage in monkey kidney cells ranges between 10^{-5} and 10^{-6}. The tissue culture LD_{50} titre is similar after 45 passages in hamster kidney cells Between the 5th and 15th MK cell passage the LD_{50} titre in mice was less than 10^{-4}, but in later passages exceeded 10^{-6}.

The establishment of this virus in tissue culture may provide supplies of large quantities of virus, free of brain tissue, for vaccine production, as well as a new route for exploring the development of an attenuated live virus vaccine for prevention of infections with RSS group viruses The successful adaptation of viruses of the Japanese B-West Nile complex to tissue culture by means of a plaque neutralization test (BHATT AND WORK, 1957) indicates that this method may be useful for strain identification, diagnosis and epidemiological studies with RSS complex viruses.

Epidemiology

Although the starting point for KFD investigations was the report of disease in monkeys, it was the involvement of the human forest population that was studied more intensively during the first few months. Man not only provided more detailed information about the manifestations of KFD virus infection but also, in the role of a sentinel animal, revealed much about mode and site of exposure, rapidity of spread and the nature and effects of the parasitism. Before considering known and potential non-human vertebrate reservoirs, arthropod vectors and origin of the virus, it may therefore be appropriate to discuss what is known of the epidemiology in human beings.

As outlined above, the first cases to be positively diagnosed by virus isolation were identified in late March 1957. Public health department records reveal, however, that patients with entirely similar symptomatology had been observed in January and February of that year and from February through April 1956. Several of these earlier cases were later shown to have developed antibodies to KFD virus

Intensive efforts to trace the epidemic to its origin uncovered what is probably the earliest case to be seen by a competent observer. This occurred in a resident of Kannur village (Figure 4) and ran a typical

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Intensive efforts to trace the epidemic to its origin uncovered what is probably the earliest case to be seen by a competent observer. This occurred in a resident of Kannur village (Figure 4) and ran a typical

clinical course which ended fatally on December 31, 1955. The patient was attended by a medical practitioner with 20 years' experience in the area who immediately recognized the disease as something new. During the subsequent epidemic he observed over 50 similar cases, in many of which it was possible to confirm the diagnosis by virus isolation or serological tests (NAIR, 1957). The presumption is therefore strong that the case in which the patient died on the last day of 1955 was in fact an instance of Kyasanur Forest Disease and the first in which the diagnosis could be satisfactorily established. As will be seen later the first reports of finding dead monkeys in the area date from the same month.

The available data suggest that during the succeeding two years the epidemic spread in all directions, though somewhat spottily, to involve a total area of approximately 70 square miles. (Figure 4). The possibility that the virus has been spread somewhat more extensively in lower animals is discussed below. The extent and character of the 1957 epidemic was followed in two ways.

First of all, the existence of suspected cases was reported daily by health inspectors, who as employees of the established public health organization visit each village about once every two weeks. In addition, a special ward was set up in Sagar Hospital for the study and treatment of presumptive cases. These procedures led to the reporting of approximately 500 cases of febrile illness initially presumed to be KFD virus infection.

The second approach was the organization of specially trained field teams which made intensive investigations of selected areas. These personnel interviewed village head men and personally examined cases previously reported to the health department. A substantial number of additional cases were brought to light in the course of these investigations Blood was drawn for virus isolation in all the acute cases encountered and for serological studies in persons giving history of a recent febrile illness which might have been Kyasanur Forest Disease.

Of 124 presumed cases studied in this way, 56 were demonstrated to be KFD virus infections by the laboratory procedures employed. This result suggests that there was considerable over-reporting and casts doubt on the total of 500 cases reported. However, the intensive search conducted by the field teams uncovered many unreported cases which were subsequently proved to be KFD. Consequently, this excessive reporting of fever cases due to other causes

may be more or less balanced by under-reporting of verifiable cases of KFD and the over-all number of KFD cases which occurred may thus actually have reached 500.

At least 6 of 56 laboratory proved cases died, a mortality rate of approximately 10%. Interestingly enough, an approximately similar rate was encountered among the fever cases reported to the health department (42 out of 466).

Convincing evidence that the human infection rate can be very high is provided by a serological study of 48 residents of Hunavalli village which has a census listed population of 92. Severe cases had been reported from this village in both 1956 and 1957. Blood studies made during the 1957 epidemic revealed that 16 of the 48 persons from whom serum had been collected had developed specific antibodies to KFD virus Nine of these 16 gave a history of illness consistent with KFD in 1957. Thus the cumulative infection rate over a two-year period was as high as 33% and the occurrence of overt disease in infected persons was at least 55% and probably a good deal higher.

Because of the uncertainty of diagnosis in many of the reported cases, further epidemiological analysis is limited to the 56 proved cases. Forty-nine of these were in males and seven in females. Only one girl and two boys under fifteen were infected and the great majority of infections is represented by 38 males and 5 females judged to be between twenty and forty years of age. A distribution of these cases by month of onset (Figure 3) shows that the epidemic

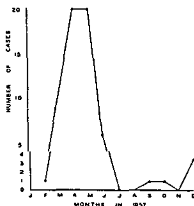


Fig 3 Occurence by month of laboratory proved cases of Kyanasur Forest Disease.

peak occurred in April and May. A few cases which ran a typical clinical course after an appropriate history of forest exposure were seen by a local medical officer (PRASANNA IYENGAR, 1957) in the last week of January and in February. It is therefore probable that the actual proportion of cases in those months is greater than appears in the graph.

The occurrence of the preponderant majority of cases in young adult males and the marked seasonal incidence strongly support the hypothesis, derived from observation of dead monkeys and the history of exposure obtained from typical human cases, that the disease is transmitted largely, if not exclusively, in the forest. It is during the period from January to the beginning of June that the villagers spend most time in the forest, and it is the young men whose occupations take them there most regularly. The monsoon rains begin in earnest in June and subside in September. The effect on the vector will be discussed later, but it is obvious that the heavy rains greatly curtail activity of the villagers in the forest and thus drastically diminish their exposure.

From the termination of the monsoon rains in September until sometime in January the people in this area are primarily engaged in paddy (rice) cultivation and most of them have little occasion to enter the forest. Virus isolations from man, monkeys and ticks in September, October and December 1957 and in January 1958 suggest that conditions for spread of the disease are favorable throughout the period. Indeed, two cases actually occurred in September and October, the first in a man who had refrained from visiting the forest since April for fear of "catching the monkey disease". In early September, with news of an apparent end of the epidemic, he visited the forest once to collect firewood. He picked up sticks to which were attached shreds of fur of a monkey long dead, and five days later suffered the onset of a typical course of KFD.

In Figure 4 the apparent spread of the infection in man has been plotted according to date of onset and residential locality in the 56 laboratory proved cases. The revenue map has been used for this purpose because it outlines areas of occupation associated with particular villages and thus shows the adjoining parts of the forest in which the 56 patients presumably were working when they were exposed. The black areas are those in which cases that occurred in 1956 were later confirmed serologically. *It can be seen that the* infection spread in all directions during the epidemic season of 1957.

All the involved localities lie within an extensive area which receives between 75–150 cm. of rainfall annually, most of it between May and October. There is an intermittent mosaic of tropical evergreen and deciduous forest surrounding oval-shaped, open lowland areas of cultivation. The villages are usually situated between these cultivated areas and forest.

In view of the apparent importance of the forest for maintenance and spread of the infection, attention may next be directed to an analysis of the disease as it appeared in the forest-dwelling monkey population. The local populations of both "black-faced" langur, *Presbytis entellus*, and "redfaced" bonnet macaque, *Macaca radiata*, are enormous. Nevertheless villagers near to where the first recorded human cases occurred deny ever having seen or heard of a dead monkey in the forest prior to early December 1955. No villager of hundreds questioned from many localities in the presently infected area had ever heard of a monkey being found dead in the forest until that time (TRAPIDO AND WORK, in press).

Presumably, under normal conditions, the carcasses of dead monkeys are rapidly removed by such carrion eaters as crows, vultures and domestic dogs Indeed many such animals were noticed to be feeding on dead monkeys during the epizootic The repeated observation of dead monkeys by villagers engaged in their usual forest activities may therefore be taken as an indication that monkey mortality had become so great as to overwhelm the natural disposal processes in the forest A total of 30 first-hand reports of dead monkeys, in addition to 14 seen by Virus Research Centre field personnel, were recorded for the first six months of 1957.

These observations, associated with repeated isolations of KFD virus form dead monkeys found in the area, suggest that wild primates played an important and perhaps essential role, not only in maintenance of the infection but also in its spread Dead monkeys were found or seen in an area which by August 1957 covered 471 square miles, as compared to the 70 square miles of the known area of human infection. In view of the previously mentioned observation that none of the local people had ever seen a dead monkey in the forest before the present epizootic, the monkey deaths were attributed to infection with KFD virus.

One qualification is necessary, however No virus has been isolated from any monkey found outside the human infected area. This may be explained by the fact that most of these monkeys were encountered

some time after death. Post-mortem histological findings in many of these monkeys were, however, consistent with those found in animals and man which are known to have died of the disease (IYER, WORK ET AL, to be published).

In any case, it is not improbable that the monkeys picked up outside the known infected area had actually contracted the disease within that area. Surviving long enough to migrate some distance, perhaps in search of water, they may also have outlived their viremia. In view of this possibility it is probably safest, in mapping the extent of the infected area, to rely on data derived from ticks, rodents and human patients.

Virus has been isolated at post-mortem from 4 langur and 3 bonnet macaque monkeys recovered from the area of human infection. Sera drawn from 37 healthy monkeys collected in the infected area and from 13 in uninfected regions were analyzed for neutralizing antibodies in mouse protection tests.

As shown in Table I, 5 out of 25 *Presbytis entellus* and 1 out of 12 *Macaca radiata* in the infected area gave evidence of previous infection. It is thus clear that many monkeys must have inapparent infections or survive overt attacks of the disease.

Not much is known about the habits and range of movement of langur and bonnet macaque monkeys. From what is known, however, it is obvious that these forest monkeys may play an important role in dissemination of KFD virus to new, unaffected areas not only by carrying infected arthropod vectors but also by circulating virus in their blood while moving through new areas.

In view of the lack of evidence for transmission of the infection from monkey to man or man to man by direct contact, and because

Table I. Neutralizing Antibodies to KFD Virus in Forest Monkeys of Shimoga District

Species	Uninfected Area					Infected Area				
	+	Inc	–	Total	+%	+	Inc	–	Total	+%
Presbytis entellus	0	0	8	8	0	5	1	19	25	20
Macaca radiata	0	0	5	5	0	1	1	10	12	8
Total	0	0	13	13	0	6	2	29	37	16

of the rough similarity of its epidemiology to that of sylvan yellow fever, it was early suspected that the virus of KFD might be carried by an arthropod vector. The absence of a distinctive canopy mosquito, the almost complete lack of diurnal mosquitoes capable of biting monkeys at ground level and the relatively slow linear spread of the disease, suggest some other arthropod. The hypothesis that ticks might be involved, as is the case with other members of the RSS complex, received early encouragement from the discovery of *Haemaphysalis* ticks on 3 of the first 4 healthy monkeys examined in April. Since that time, and somewhat at variance with the general opinion that their grooming habits tend to keep monkeys relatively free from such ectoparasites, larval and nymphal ticks have been found on almost all monkeys collected in the infected area except during the rainy season. During the latter period only two of 11 monkeys were found to be infested.

With diversion of attention from mosquitoes to ticks and mites as possible vectors, arrangements were made for recovery of ectoparasites not only from monkeys but also from the trees and floor of forest areas known to be infected. Pieces of cotton flannel, one meter square, dragged over the forest floor collected large numbers of larval and nymphal ticks of three genera, *Haemaphysalis*, *Ixodes*, and *Amblyomma*. They could not be identified according to species but were divided into groups by genus for trituration and inoculation into mice in attempts to isolate virus. Two pools of larval and nymphal *Haemaphysalis* ticks obtained during the first three days of collections initiated in Kyasanur Forest on the 15th of April, yielded virus (TRAPIDO ET AL, to be published). It is now known that some of the nymphs in these pools were *Haemaphysalis spinigera*.

Since these two initial instances, all except two isolations of KFD virus from ticks collected in forest drags and from low, leafy vegetation, have been from *Haemaphysalis spinigera* adults; two of the pools which yielded virus were collected in July, eight in August and four in September (including one pool of *H. turturis* and one of *H. papuana*). *H. spinigera* adults were the predominant forms in the collections in July and August (VARMA ET AL, in press). Of the identifiable ticks collected from monkeys in the infected area, *H. spinigera* has been most common and nymphs of this species were also detached from man on four occasions. Three of these were actually engorged with blood. The predominant identifiable *Haemaphysalis* tick collected from monkeys has been *H spinigera*.

Genera and species of ticks which have been inoculated without isolation of virus include: *Ixodes* spp., *H. cornigera typica*, *H. cuspidata*, *H. aculeata*, *H. wellingtoni*, *H. minuta*, *H. formosensis*, *Boophilus microplus*, *Rhipicephalus sanguineus*, *R. haemaphysaloides*, *Dermacentor auratus*, *Amblyomma testudinarium* and *A. integrum*.

The composition of these forest tick collections has varied seasonally. In May, before the onset of the heavy monsoon rains, the yield of *Haemaphysalis* species consisted almost entirely of larvae and nymphs. The proportion of adults increased during June and reached a peak in July and August. With subsidence of the rains in September, adults diminished and the numbers of larvae increased enormously (VARMA ET AL, in press). As the dry season progressed the ratio of nymphs to larvae increased, as it did also in the collections obtained from monkeys during the same period.

The close relationship of KFD virus to the Russian Spring Summer Encephalitis tick-borne viruses which exist in an *Ixodes* tick-forest rodent cycle in Siberia (SILBER AND SOLOVIEV, 1946), led to consideration next that forest rodents and small mammals might play a role in maintaining a silent reservoir of KFD virus infection. There had been no reports of an epizootic in small forest mammals and as yet no unusual number of dead rodents have been found in the infected area.

In order to explore the possibility of a rodent reservoir fully, systematic trapping of small mammals in and outside the infected area was undertaken. Seven species of small mammals have repeatedly appeared in traps in both areas; *Suncus murinus*, *Funambulus tristriatus tristriatus*, *Mus booduga*, *Rattus rattus wroughtoni*, *R. r. rufescens*, *R. blanfordi*, *Tatera indica hardwickei*. In contrast to the large number of *Haemaphysalis* ticks found in forest drags and on wild monkeys, the ticks on the small mammals have been almost entirely larval and nymphal *Ixodes*. Very few have been *Haemaphysalis*. This finding implies that there may be separate cycles of transmission in small forest mammals and wild monkeys (TRAPIDO AND WORK, in press). Most of the few *Haemaphysalis* were taken from *R. r. wroughtoni*, the species collected in larger number than all the others combined. Some were collected (actually in higher percentage) from *Funambulus t. tristriatus*, the tree-climbing squirrel.

No animals were trapped in Kyasanur Forest during September, November and December, 1957, so what ticks infested the small mammals in those months, relative to the enormous increase in

Haemaphysalis larvae and nymphs collected from the ground, is not known. Incomplete results of collections made in other infected forest areas indicate that there was relatively little increase in *Haemaphysalis* ticks collected from these small mammal species.

The trapped mammals were bled for serum to test for neutralizing antibodies. Results of mouse neutralization tests of these sera against KFD virus are given in Table II.

Although study of infection and specific antibody development has not been accomplished in the laboratory, the difference in antibody incidence between infected and uninfected areas indicates the probable specificity of these tests. It therefore appears that the small mammals in Kyasanur and neighboring infected forest areas sustain infection on occasion and that a large number survive. The percentage of infection in *Rattus* species is small but significant, considering the number of animals involved. *Funambulus* shows a much higher infection rate. This high incidence of antibodies and the previously mentioned higher percentage take of *Haemaphysalis* ticks from this animal along with the highest percentage score for *Ixodes* larvae and nymphs may provide an important epidemiological clue.

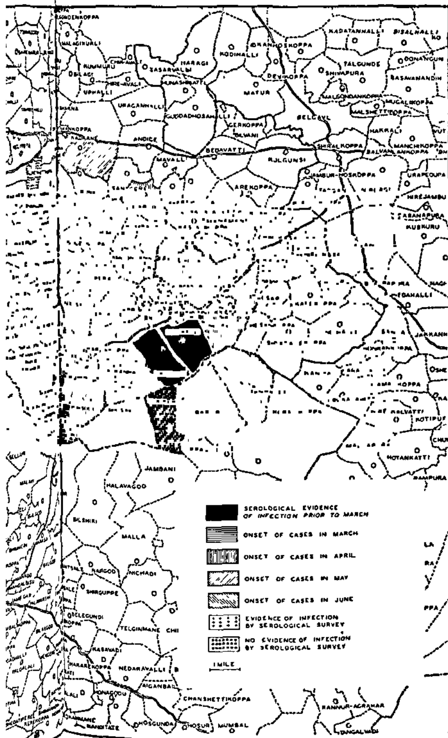
Evidence for believing that the occurrence of Kyasanur Forest Disease in the presently known infected area of Shimoga District is

Table II Neutralizing Antibodies to KFD Virus in Sera of Small Forest Mammals of Shimoga District

Species	Uninfected Area					Infected Area*				
	+	Inc	−	Total	+%	+	Inc	−	Total	+%
Rattus rattus wroughtoni	0	1	51	52	0	13	18	205	236	5 5
Rattus rattus rufescens	0	0	4	4	0	0	0	26	26	0
Rattus blanfordi	0	0	5	5	0	2	4	70	76	2 6
Mus booduga	0	0	0	0	0	0	0	13	13	0
Funambulus t tristriatus	0	0	2	2	0	11	4	16	31	35
Tatera indica hardwicket	0	0	11	11	0	1	1	3	5	20
Suncus murinus	1	0	12	13	8	9	1	49	59	15
Total	1	1	85	87	1%	36	28	382	446	8%

* Three forest localities in known infected area Kannur, Hosur and Kyasanur Forest

Fig 4. Distributional Occu ence of Human Kyasanur



e of Human Kyasanur Forest Disease cases in 1957 by Month of Onset.

due to a recent introduction of the virus has been presented previously. The question as to the site of origin of the virus and mode of introduction into the Shimoga District naturally follows. At least two possibilities have been considered: transmission by human agency or by migratory birds.

An incubation period of 3–8 days and a viremia of at least 10 days provide a period of time long enough for the migration of people from any one locality to any other in India. There is considerable coastal traffic from Saurashtra, where there is serological evidence that the virus exists, to the Malabar coastal ports. There is traditional migration of laborers from the coastal plain to work in the paddy fields and areca palm plantations in the Malnad of Mysore.

The second, and at present more acceptable, hypothesis is that the virus may be carried by the most ubiquitous migrants of all, the avian species, which either have some seasonal local migration or winter in the area after having flown from as far away as Siberia, where various strains of RSS virus have been found in many localities. *Haemaphysalis* and *Ixodes* ticks have been collected from various avian species in Russia (Silber and Soloviev, 1946; Olenov, 1954; Pospelova-Shtrom 1936; Grobov, 1946). KFD virus isolations from *Haemaphysalis spinigera* and *H. turturis* have been detailed above. A viremia in such migrants, chronically recurring or of sufficient duration, could have brought the virus into a new but similarly tick-infested environment.

The chancier possibility of a "leap-frogging" of the infection from area to area via different birds, either transporting infected ticks or viremic infection, might explain the rare and spotty distribution of the presently known infected areas of India.

It is also possible that the virus has been present in several localities of India and is only now being recognized because facilities have become available for isolating and identifying such agents. Furthermore, the virus could be widely distributed in birds and lower mammals but have come only recently under observation because some unusual combination of circumstances has led to the infection of monkeys and man. It has even been suggested that RSS virus may have originated in India or some other tropical area from which it was long ago carried to the temperate zone of Asia where it was first recognized by the Russians. In this connection it should be recalled that the TP 21 strain of RSS virus has recently been isolated from *Ixodes granulatus* in Malaya (Smith, 1956).

As a preliminary to more extensive exploration of avian introduction, efforts have been made to determine whether resident birds play a role as host to the virus and or its vectors. It has been demonstrated that the grey jungle fowl, *Gallus sonnerati*, which frequents ground and forest scrub, is host to at least three species of *Haemaphysalis* ticks, including *H. spinigera* which has been collected in numbers from each of four of these birds. More than six hundred *Haemaphysalis* ticks were collected from a single peafowl, *Pavo cristatus*, obtained in the heart of the infected area. *H. spinigera* nymphs have also been collected from an Indian robin, *Saxicoloides fulicata*, which was perching in a thick clump of bamboo, and a white-throated ground thrush, *Geocichla citrina cyanotis*, a bird which spends most of its time on the ground in the forest. Neutralizing antibodies have been demonstrated in serum from the grey jungle fowl and the golden-backed woodpecker, *Brachypterus benghalensis puncticlolis*, which has foraging habits strikingly similar to those of the squirrel, *Funambulus*, and can often be seen in the same trees.

The accumulating circumstantial evidence that birds may play a role in the transport of infected ticks, if not indeed of circulating virus as well, demands more extensive investigation of the avian species which: (1) are locally migratory from one site in India to another; and (2) breed in RSS endemic areas north of the Himalayas and funnel through Kutch, Saurashtra and Rajasthan to spend the winter months in India.

Conclusion

At this writing, barely nine months have passed since recognition of the RSS viral etiology of Kyasanur Forest Disease. Unique situations and associations in the epidemiology of this infection have already been suspected or recognized, but complete annual cycles of data are not available on any aspect. The information presented has, therefore, been qualitative, spotty and incomplete; some of it points substantially to certain facts, but much of it is only suggestive of trends and supports working hypotheses.

The attention of Virus Research Centre investigators was originally attracted by the occurrence of a fatal epizootic in two species of forest monkeys in tropical evergreen and deciduous forested areas of Shimoga District, Mysore State. Associated with this epizootic was a prolonged, febrile, prostrating disease in human beings. Strains of

virus identified as belonging to the Russian Spring Summer complex were isolated from monkeys and man. The human disease was characterized by sudden onset of fever, headache, conjunctival inflammation, and back and limb pains, followed by vomiting and diarrhoea. Epistaxis, hematemesis, hemoptysis, melena and bloody stools, indicative of hemorrhage, occurred in the more severe cases. The fever continued for five to fourteen days. Occasionally a second bout of fever occurred in the third or fourth week after onset. The disease was prostrating and led to slow convalescence but complete recovery without sequelae. No specific sign of neurological lesions in the central nervous system was noted. An invariable leucopenia, no abnormal changes in the spinal fluid and histological evidence of damage in the liver and kidneys indicated a visceral rather than a neurological disease.

The disease occurred in young adults, predominantly males, exposed in the forest during the dry season from January to June. A high [illegible] simulates Omsk Hemorrhagic Fever (CHUMAKOV, 1948; BILIBIN, 1950) which is now known to be caused by a RSS complex virus. Slight clinical differences, involvement of monkeys and occurrence in the tropics led to naming the infection Kyasanur Forest Disease.

In contrast to the difficulty with which Omsk Hemorrhagic Fever virus was adapted to mice (CHUMAKOV, 1948), KFD virus was readily isolated by intracerebral inoculation of blood serum and other tissue of monkeys and man into infant and adult white mice. There is growing evidence that *KFD virus is immunologically closely related* to RSSE virus, although probably distinguishable from it. The virus has been isolated from the blood serum of man from two days before to ten days after onset of the disease. Infection has been induced in rhesus as well as bonnet macaque monkeys by IC and IP inoculation; the viremia usually lasts about seven days. Suckling hamsters are susceptible to infection, and adult hamsters and cotton rats develop specific antibodies after inoculation. The virus has been isolated by direct inoculation of infected wild monkey serum into chick embryo and hamster kidney tissue culture cells. Passage of the virus in tissue culture produces marked cytopathogenic effects in monkey kidney and hamster kidney cell cultures.

The virus has been isolated repeatedly from *Haemaphysalis spinigera* ticks collected by drags on the floor of infected forest areas.

Haemaphysalis ticks, including *H. spinigera*, have been collected in large numbers from wild forest monkeys during the dry season and occasionally from small forest mammals, particularly the tree-climbing palm squirrel, *Funambulus t. tristriatus*. However, most of the ticks collected from small forest mammals have been larval and nymphal *Ixodes*, species undetermined. Infection of these mammals is indicated by presence of neutralizing antibody against KFD virus in species of *Rattus*, the squirrel, and a shrew collected in the infected areas, as compared to negative evidence of infection in representatives of the same species collected outside the presently known infected area.

Haemaphysalis ticks have also been recovered from ground and bush-dwelling birds in Kyasanur Forest. *H. spinigera* has been found on all tick-infested birds collected so far. The role of avian species in the maintenance of a tick-bird-virus cycle and in dissemination of the virus remains to be worked out, but these preliminary clues indicate a possible mechanism for introduction of the virus into this newly infected area from elsewhere in India if not from north of the Himalayas. Certainly enough evidence exists by commonplace finding of *Haemaphysalis spinigera* on birds in Kyasanur Forest and repeated isolations of KFD virus from this tick species to suggest that tick-infested birds may play an important role in the spread of the virus to neighboring susceptible forest areas in India. It is obvious, however, that there is still much to be learned, not only about the maintenance of the virus in non-human vertebrates and arthropods, but also about the pathogenesis, treatment and prevention of the disease in man.

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DIRECT VIRUS ANTIBODY FLOCCULATION REACTIONS

By WILSON SMITH

I. Introduction

For many years the only serological reaction available for the investigation of virus infections was virus-neutralisation in which recognition of the occurrence of an interaction between viral antigens and their homologous antibodies in immune sera necessitates the use of animals which are susceptible to experimental infection with the virus in question. This was not due to any want of appreciation by early workers of the impetus which virology would receive by application of those in-vitro serum reactions which had long been used to such good effect by bacteriologists and cytologists. The reason lay chiefly in the lack of knowledge, and of the technical equipment, required in order to separate viruses and their products from infected tissues, so as to obtain them in the form of relatively pure and concentrated antigenic material. It is true that complement fixation is often demonstrable with crude antigenic material, being much less

influenced than are agglutination and precipitation by the presence of extraneous substances. It is not surprising therefore that complement fixation is now fairly widely used in laboratory diagnostic tests whereas the direct virus-antibody reactions have been very largely neglected. The term 'direct' is used to denote these reactions between antigenic material and serum antibodies which become visibly manifest without the introduction of any indicator system. In this sense both virus neutralisation and complement fixation are indirect reactions, and suffer from the limitations inherent in any system in which extraneous variables must be introduced In a few virus diseases it has now been shown that by the application of modern methods of virus cultivation, purification and concentration, the direct reactions, analogous with bacterial agglutination and precipitation, can be employed both as analytic research tools and as practical diagnostic tests. In the near future their extension to a large number of other virus infections can be confidently expected.

WILSON AND MILES (1946) differentiate bacterial agglutination and precipitation as reactions involving respectively antigens which form part of the structure of the bacterial cell and antigens in colloidal solution. The two types of reactions have quite distinctive characteristics There is no doubt that, with the appropriate virus reagents, both true agglutination and true precipitation can be shown to occur in certain special cases but in many instances the type of reaction is indeterminate. This is especially true of the smallest viruses which approach molecular size. For this reason the term 'flocculation' is to be preferred except when it is clearly possible to draw a distinction between agglutination and precipitation.

The occurrence of flocculation reactions and their possible applications were recognised equally by early workers in both animal and plant virology. Until recently, progress in the field of animal virology lagged behind, chiefly because large quantities of relatively pure virus material are much easier to obtain from infected plants than from the tissues of infected animals This position, however, has been rectified to some extent by modern developments of cell culture techniques for the growth of viruses, with the result that rapid progress is now being made in the study of flocculation reactions with some of the animal viruses. In the present article attention will be confined to work with animal viruses, especially those causing diseases of man, plant and bacterial virus infections being left out of consideration.

The more important human virus infections in which flocculation reactions of one sort or another have been reported are variola, vaccinia, influenza, herpes, yellow fever, psittacosis, lymphogranuloma inguinale, lymphocytic choriomeningitis and poliomyelitis. They have also been applied in studies of several virus infections of lower animals such as fowl pox, foot and mouth disease and vesicular stomatitis. Most of our knowledge of the factors involved in the reactions themselves, however, has been derived from systematic studies with the pox viruses, influenza and poliomyelitis, and it is with these that the present article is chiefly concerned.

II. *Virus Flocculating Antigens*

(*A*) *Crude Tissue Suspensions*

Flocculation sometimes occurs when crude suspensions of the tissues from an infected animal are mixed with a specific immune serum. This was demonstrated with vaccine lymphs over fifty years ago (TANAKA, 1902; FREYER, 1904). Many workers, however, were unable to elicit the reaction with similar reagents and attributed flocculation in such systems to the presence of extraneous factors such as contaminating bacteria. Moreover, in other virus infections attempts to obtain specific flocculation with crude tissue suspensions have, with few exceptions, been reported as complete failures. There are good reasons for these discrepancies. In the first place a critical minimum concentration of virus, or antigenic component of the virus, must be present for the formation of visible aggregates. It is doubtful whether this critical concentration is ever attained in the course of some virus diseases and in others it is probable that antigen concentrations fluctuate so that the time of harvesting material becomes a determinant factor. Furthermore, the number of virus particles per unit volume of suspension, as estimated by infectivity titres, may be no index of the total amount of viral antigen present even in the case of one particular virus infection. With different infections the relative sizes of the viruses are obviously important and it is probably significant that flocculation studies were for long confined almost exclusively to the pox viruses which are amongst the largest animal viruses known. Recent work with small viruses has shown the necessity of using concentration methods for the production of flocculating suspensions.

The exact nature of the antigens responsible for flocculation of crude tissue suspensions cannot be determined because the reaction may involve, at one and the same time, several distinct antigen-antibody systems. For example vaccinia convalescent sera contain four separate specific antibodies, any one of which will agglutinate elementary body suspensions, and three of which will give a precipitation reaction with the homologous antigenic components extracted from the virus particles (L, S and NP antigens). Flocculation of crude vaccine lymph gives no indication of which system is operative or whether only one or all together are concerned.

In spite of these limitations the flocculation reaction with vaccine lymph as the antigenic material has been shown to be applicable as a diagnostic technique. This was first unequivocally demonstrated by GORDON (1925) but it was by the subsequent extensive investigations of BURGESS, CRAIGIE AND TULLOCH (1929) and CRAIGIE AND TULLOCH (1931), that several points of cardinal importance were firmly established. These workers definitely refuted the criticism that secondary bacterial invaders might be responsible for the reaction. They concluded however that, although the flocculation observed was a specific reaction, the antigen involved could not be the intact virus particles but represented a soluble component of the virus. In their hands the flocculation resembled a precipitation rather than an agglutination. One of their most important findings was that the presence of impurities might inhibit flocculation so that, for consistency of results, ether extraction of the vaccine lymph was necessary. This clearly pointed to the presence in crude lymph of flocculation inhibitors which have assumed great interest and importance in more recent work.

Early work with influenza viruses pointed in the same direction. MAGILL AND FRANCIS (1938) found that lightly centrifuged suspensions of the lungs of infected mice would flocculate when mixed with some human sera, especially with sera of convalescent patients. The reaction occurred within an hour at 37°C but might not become visible until the mixtures were shaken. The antigens were reactive only up to ½ or ¼ dilution, but attempts to increase their potency by purification and concentration, by means of centrifugation, were unsuccessful. Sera were considered strongly positive if they gave flocculation at dilutions of $^1/_8$ or $^1/_{16}$. Heating either the antigen or serum at 56°C for 1 hour abolished reactivity. The authors adduced evidence of the specificity of this reaction but expressed some doubt

The more important human virus infections in which flocculation reactions of one sort or another have been reported are variola, vaccinia, influenza, herpes, yellow fever, psittacosis, lymphogranuloma inguinale, lymphocytic choriomeningitis and poliomyelitis. They have also been applied in studies of several virus infections of lower animals such as fowl pox, foot and mouth disease and vesicular stomatitis. Most of our knowledge of the factors involved in the reactions themselves, however, has been derived from systematic studies with the pox viruses, influenza and poliomyelitis, and it is with these that the present article is chiefly concerned.

II. Virus Flocculating Antigens

(A) Crude Tissue Suspensions

Flocculation sometimes occurs when crude suspensions of the tissues from an infected animal are mixed with a specific immune serum. This was demonstrated with vaccine lymphs over fifty years ago (TANAKA, 1902; FREYER, 1904). Many workers, however, were unable to elicit the reaction with similar reagents and attributed flocculation in such systems to the presence of extraneous factors such as contaminating bacteria. Moreover, in other virus infections attempts to obtain specific flocculation with crude tissue suspensions have, with few exceptions, been reported as complete failures. There are good reasons for these discrepancies. In the first place a critical minimum concentration of virus, or antigenic component of the virus, must be present for the formation of visible aggregates. It is doubtful whether this critical concentration is ever attained in the course of some virus diseases and in others it is probable that antigen concentrations fluctuate so that the time of harvesting material becomes a determinant factor. Furthermore, the number of virus particles per unit volume of suspension, as estimated by infectivity titres, may be no index of the total amount of viral antigen present even in the case of one particular virus infection. With different infections the relative sizes of the viruses are obviously important and it is probably significant that flocculation studies were for long confined almost exclusively to the pox viruses which are amongst the largest animal viruses known. Recent work with small viruses has shown the necessity of using concentration methods for the production of flocculating suspensions.

pox diagnosis for several reasons. The elementary body suspensions had to be derived from infected calves or rabbits and the technique used by LEDINGHAM involved the microscopical examination of hanging drops kept in sealed slide chambers for at least 48 hours. Also in smallpox the rise of serum agglutinins is so slow that, long before they become detectable, diagnosis can be firmly established, either clinically or by other serological methods.

The practicability of using purified virus suspensions both for antigenic analysis and diagnosis emerges from the recent work of BELYAVIN (1955, 1956) with viruses of the influenza group. He showed that high titre allantoic fluids may or may not give visible flocculation with the sera of immunised rabbits and concluded, from analogy with the phenomena of haemagglutination inhibition that such fluids must contain variable amounts of a flocculation inhibitor. Removal of inhibitor, especially if accompanied by some measure of virus concentration, yields antigens giving regular and reproducible flocculation. Such purification and concentration is especially easy with the influenza viruses because of their adsorbability on, and spontaneous elution from, human or fowl red blood cells Using this technique satisfactory flocculating virus suspensions have been obtained for influenza viruses of types A and B, the A prime sub type, and many strains within each type; also for the viruses of mumps and Newcastle disease of fowls. The strains within each type show de- [illegible]

Partially [illegible]

[illegible]

size of the virus particles even high titre virus culture fluids require from fifty to a hundred fold concentration. It is not known as yet how important the removal of impurities may be. In this case purification by red cell adsorption and elution is not applicable so that recourse must be had to differential centrifugation. The antigens thus obtained by SMITH AND HIS COLLABORATORS certainly contain relatively large amounts of non-viral protein. In the author's opinion, however, success was largely dependent upon the use of cell culture fluids with their high virus titres, coupled with their very low content of extraneous material, as compared with tissue suspensions obtained

because its intensity did not parallel the viral content of the antigen, as determined by infectivity tests in mice. For reasons stated above we would no longer regard this as a valid objection. More cogent reasons for doubt were the occurrence of flocculation with convalescent sera from cases of illness which were not influenza and also, in some fractionation experiments, in the complete absence of detectable virus. Neither of these reasons, however, is in any way conclusive.

HENLE AND CHAMBERS (1941) were unable to confirm this work with infected mouse lungs but demonstrated flocculation with allantoic and amniotic fluids from infected embryonated eggs. They offered the interesting suggestion that failure of high titre mouse lung suspensions might be due to mechanical hindrance of specific flocculation by normal lung particles, a possibility which could now very easily be put to experimental proof.

(B) *Purified Virus Suspensions*

Recognition of the particulate nature of vaccinia virus and its microscopical demonstration in the form of stained elementary bodies by PASCHEN (1913) directed attention to the possibility of their agglutination by specific immune sera. PASCHEN himself reported such agglutination and GINS (1922) demonstrated microscopic flocculation of virus suspensions with high dilutions of immune antivaccinia sera by D. G. illumination. No significant advances however were made until LEDINGHAM (1931, 1932, 1933), CRAIGIE (1932) and CRAIGIE AND WISHART (1936) carried out systematic investigations. LEDINGHAM working with both vaccinia and fowl pox viruses found that the starting material must be rich in elementary bodies, that preliminary ether extraction and subsequent concentration of the elementary bodies by high speed centrifugation were necessary, and that impurities must be removed from the concentrates by differential centrifugation. Antigens thus prepared gave agglutination with convalescent and hyperimmune sera in high dilution. The specificity of the reaction was established beyond question. The work of CRAIGIE AND WISHART concerned more especially the soluble antigens of the virus and is referred to below.

In spite of the scientific importance and theoretical applicability of this work it failed to find practical application in the field of small-

that many viruses contain antigens which could be extracted in soluble form. Apart from their possible use as precipitinogens in diagnostic serology, pure antigens in soluble form are particularly suitable for investigations of immunological skin reactions.

III. Virus-Flocculating Antibodies

The antigenic analyses of vaccinia virus and of the influenza group of viruses have shown that specific immune sera may contain antibodies of many different kinds produced under the stimulus of different antigenic components of the virus. These may show independent quantitive variability in immune sera of different animal species, different animals of the same species and different samples from the same individual. Not all these specific antibodies are capable of flocculation with either whole virus suspensions or soluble virus antigens. In the agglutination of pure virus suspensions only the surface antigens are involved, and for the formation of antigen-antibody lattices with soluble antigens it is necessary for both antigen and antibody molecules to have at least more than one combining site. There is conclusive evidence that some vaccinial and influenzal immune sera contain univalent antibodies—so called blocking antibodies—which can combine with virus but cannot flocculate, and this may account to some extent for the gross lack of parallelism between virus neutralisation and flocculation titres. It may also acccount for the fact that some poliomyelitis hyperimmune monkey sera of high virus neutralising titre give flocculation of quite a different type, and to much lower titre, than do rabbit and human sera of comparable neutralising potency. The production of potent flocculating sera in experimental animals therefore depends on methods evolved empirically. Likewise the clinical and diagnostic significance of flocculation with immune sera can only be determined by empirical tests of the correlation between flocculation titres and other immunological data. The late appearance of agglutinins in smallpox has already been mentioned and it is established that none of them are effective in virus neutralisation. On the other hand, the scanty evidence at present available suggests that, in poliomyelitis, flocculating antibodies appear in the blood serum very early after the onset of infection, though their possible significance in protection is not yet known.

from infected animals. There is as yet no clear evidence of the occurrence of flocculation inhibitors similar to those involved in influenza virus flocculation, but work in progress in the writer's laboratory *suggests that they may exist and may play an important role in the* reaction.

Because of the relative impurity of the poliomyelitis antigens hitherto used, formal proof that the reaction involves an agglutination of virus elementary bodies is lacking. Indeed it is most improbable that the bulky floccules consist entirely of virus and molecules of antibody. But the close correlation between the flocculation potency and the virus concentration of the antigens indicates that a direct agglutination of virus particles is probably the basic reaction. This conclusion is supported by the fact that much of the extraneous protein can be removed by chemical precipitation without reducing flocculating potency (unpublished observations).

(C) Soluble Flocculating Antigens

The precipitinogens extractable from vaccinia virus have already been mentioned The L. S. complex (CRAIGIE, 1932; CRAIGIE AND WISHART, 1936), consists of two distinct antigenic components which can be dissociated, either of which will combine with its homologous antibody when added to an immune serum, but for formation of a visible precipitate the intact complex is necessary. A third precipitinogen is the nucleoprotein (NP) antigen of SMADEL, RIVERS AND HOAGLAND (1942). These must all form part of the surface structure of the elementary bodies for the latter are agglutinated by each of the three corresponding antibodies. The Koktopräzipitinogene of TORIKATA (1917) probably represents the heat stable component of the L. S. complex, as does also the precipitinogen extractable from the testes of rabbits infected with a neurotesticular strain of the virus (SMITH, 1932).

Flocculation reactions have been reported from time to time in several other virus infections, notably in yellow fever, psittacosis, lymphogranuloma, lymphocytic choriomeningitis, herpes and dog distemper, but little is known about the nature of the reactive antigens concerned. In some cases, at least they may represent tissue products of the infection rather than components of the viruses themselves (HUGHES, 1933). None the less it is reasonably certain

half an hour and increases in degree, with extension to higher dilutions of both reagents, over a period of a few hours. The floccules are easily visible by naked eye against a black background, their size varying according to the concentration of the reagents. Results may be read at any arbitrarily selected time and expressed in any arbitrary notation. BELYAVIN uses the classical notation of bacterial agglutination with *Total* (T), *Standard* (S+, S, S−) and *Trace* (Tr+, Tr, Tr−) to express all degrees of particulation from complete sedementation of large floccules, with clear supernatant fluid, to minute aggregates only just visible with the aid of a hand lens. The reaction is indeed strictly comparable with a Widal reaction in its simplicity of technique, speed and visual appearances. Its development over a period of 5 hours with standard reagents is shown in table 1, kindly supplied by BELYAVIN. Different types of floccules are produced by different virus strains; some strains give coarse flocculation of the 'H' type of bacterial agglutination, others give granular flocculation of the 'O' type [see figs. 1 and 2]



Fig 1 Influenza Virus Flocculation by macroscopic tube method
+ reaction PR8 virus with specific rabbit antiserum
− reaction PR8 virus with normal rabbit serum

Fig 2 Flocculant (H) and Granular (O) types of specific influenza virus flocculation

IV. Flocculation Techniques

All the techniques which have been used in bacteriological agglutination and precipitation work have been applied at one time or another in virus flocculation. Some of them however have done little more than serve to show the occurrence of a reaction, often of doubtful specificity, without any indication of the complexity of the factors involved. For example ring precipitation by the layering of antigen solution over serum was used by TOMARKIN AND SUAREZ (1917) and SMITH (1932), microscopic hanging drop flocculation by LEDINGHAM (1933) and dark ground microscopy by GINS (1922). Many attempts have also been made to attach viruses to carriers such as bacteria, collodion particles, red cells and particulate dyes, with the object of rendering virus agglutination more easily visible; reactions with such complexes however cannot be regarded as direct reactions as defined above. The techniques used in more recent work which have proved suitable for investigations of the reactions *per se* fall into three categories; macroscopic tube flocculation, micro techniques involving sampling for dark ground microscopical observation and gel diffusion techniques. Each of these has its particular advantages and disadvantages.

(A) Macroscopic Tube Flocculation

The full potentialities of this method have been revealed by BELYAVIN'S studies of influenza virus flocculation. It is in all respects similar to the standard methods of diagnostic bacterial agglutination, in which equal volumes of antigen and serum dilutions in physiological saline are mixed in Dreyer agglutination tubes and incubated in a water bath at 37°C The possibility of using higher incubation temperatures to speed up the reaction has not yet been explored, nor have the effects of different Hion concentrations. From the occurrence of inhibition zones obtained by CRAIGIE with vaccinia at 55°C and their absence in LEDINGHAM'S tests at room temperature, and also from the abolition of flocculability of influenza virus antigens by heating at 56°C, reported by MAGILL AND FRANCIS, it is probable that temperature of incubation may be a decisive factor in the case of each particular virus. The induction of convection currents by only partial immersion of the mixtures in the water bath is also a factor of some importance. With influenza viruses flocculation usually begins within

mixtures, certainly within half an hour, and thereafter increases in degree and extends in range, in the same manner as BELYAVIN's influenza flocculation described above. An incubation period of four hours has therefore been generally adopted as standard procedure. The few monkey sera tested and some human sera, however, flocculate much more slowly and may even require overnight incubation for reactions to become visible. The practical feasibility of adopting such prolonged incubation depends upon the stability of the antigen preparations employed and this factor has not yet been satisfactorily controlled for, whilst some batches of antigen show no spontaneous flocculation after 24 hours incubation, other batches, prepared in exactly the same way, do so. Investigations under way in the writer's laboratory indicate that this difficulty will soon be overcome.

The flocculation within a fairly wide range of dilutions is quite striking and very easily detected, a mere glance usually sufficing to record the hanging drop sample as positive or negative [Fig. 3]. Near the end points of the reaction, however, the particulate nature of the antigens themselves makes reading more difficult and careful comparison with the control mixtures is necessary.

The micro tube technique therefore, useful as it has proved to be, cannot compare with the macroscopic method for simplicity, reliabi-

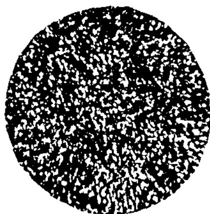


Fig. 3. Poliomyelitis virus floculation by micro tube method
\+ reaction Type 1 (Brunhilde) Antigen with type 1 rabbit antiserum
− reaction Type 1 (Brunhilde) Antigen with type 3 (Leon) antiserum

The same technique was shown to be applicable to poliomyelitis virus flocculation by SMITH, SHEFFIELD, LEE AND CHURCHER (1956). Investigations of the dynamics of the reaction in this case, however, have not yet been made because of the difficulties involved in producing adequate quantities of antigen. This indeed imposes a serious limitation on the immediate extension of this method to other viruses, for the minimal quantities of each reagent per tube to give satisfactory flocculation is 0.25ml; the use of smaller quanities in tubes of narrower bore affects adversely both the speed and visibility of the reaction. Present indications are, however, that this macroscopic tube flocculation will eventually be found applicable to many viruses and will come to hold a place in diagnostic virology comparable with the agglutination reaction in bacteriology.

(B) Micro Tube Method

The difficulty of obtaining sufficient quanities of poliomyelitis virus antigens for investigations by the macroscopic tube method led SMITH AND HIS CO-WORKERS to evolve a technique by which the total volumes of reaction mixtures are reduced to 0.04ml. per tube. For full details reference should be made to their paper (1956b), but the essential features are as follows Antigen-antibody mixtures are made in Dreyer agglutination tubes, by means of dropper pipettes calibrated to deliver 50 drops per ml. They are incubated at 37°C within a humidity box to prevent evaporation. Observation of flocculation is by low power dark-ground microscopy of hanging drop preparations, sampling being by platinum loop. For the purpose a low power dark-ground condenser of long focal length is required.

This technique has proved to be sufficiently accurate and rapid for many types of investigation. Full chess board titrations with antigens of each poliomyelitis virus type against three or four sera can be completed in the course of a day. It is particularly suitable for screening tests and may eventually find useful application in routine diagnostic work. So far as the writer is aware however, it has not been used in work with viruses other than poliomyelitis viruses and, even with these, experience is insufficient as yet to define the optimal conditions and its limitations for practical use. In chess board titrations with the sera of immunised rabbits and with many human acute and convalescent sera, flocculation occurs rapidly in optimal

which are often completely obscured in the classical methods of agglutination and precipitation unless laborious cross absorption techniques are employed The plate technique has the particular advantage that several different antisera or antigen preparations can be included in a single test for direct comparison of their reactions. By careful spacing identity of antigens shared by different virus strains is revealed by fusion of their precipitation bands at their points of juxtaposition.

The first application of gel diffusion flocculation in virology was by JENSEN AND FRANCIS (1953) who worked with influenza viruses of types A, B and A prime. They were unable to decide whether the virus particles or soluble antigens were involved From the fact that several flocculation bands were obtained and the interesting observation that convalescent ferret sera and human sera gave fewer bands than did sera of immunised rabbits, it may now be concluded that multiple antigens were concerned, some of which at least were probably soluble components With the pox viruses it is possible that only soluble antigens give diffusion-flocculation, for GISPEN (1955) failed to elicit reactions with purified vaccinia elementary body suspensions This may, however, have been due to the use of an agar gel which was too stiff to allow the diffusion of relatively large virus particles and the matter calls for reinvestigation. GISPEN's work draws attention to two phenomena which impose serious limitations on gel diffusion flocculation, namely that only a single precipitation band will be formed by two distinct antigens and their corresponding antibodies, if their diffusion rates happen to coincide, and that conversely two bands may be produced by a single antigen-antibody system if immunologically identical haptens of different molecular sizes form part of the intact antigen For complete antigenic analysis therefore the introduction of fractional absorption methods is necessary.

A still more serious limitation which is likely to preclude the use of these macroscopic diffusion methods for routine diagnostic tests is the slow speed of the reactions. Time of appearance of precipitation depends of course upon the distance between the reservoirs of antigen and antiserum, but with the spacing necessary to give satisfactory separation of multiple zones, most workers find that incubation for several days is required This is in sharp contrast to the speed of flocculation in liquid mixtures reported by BELYAVIN and by SMITH and his co-workers The microscopic method, outlined below, however, appears to remove this drawback

lity and accuracy. It has one further serious limitation. For studies of the dynamics of flocculation reactions, continuous observation of the whole range of reaction mixtures is essential. This of course is not possible with any techniques which involve serial sampling and microscopical examination.

(C) *Gel Diffusion Techniques*

(1) Macroscopic Methods

Gel diffusion techniques for the study of virus precipitation reactions are all modifications and adaptations of the methods originally introduced by OUDIN (*1946*), OUCHTERLONY (*1948*) and ELEK (1948/49) for the antigenic analysis of bacterial toxins and other soluble precipitinogens. They are based on the principle that when antigen and antibody molecules meet, by diffusion from separated loci in an agar gel, their interaction is often manifested by the formation of a visible precipitate. Various refinements are adopted by different workers but the two important macroscopic methods available are the tube method and the plate method. In the former, antigen and antiserum are separated by an agar column in a tube of suitable bore. In the latter the agar gel is contained in a Petri dish and holes to hold the reagents are made by means of a mould which ensures their uniform spacing. Consistency, clarity and pH of the agar gel and temperature are important factors, details of which may be found in the various publications cited. Such factors being standardised, however, the rate of diffusion of antigen and antibody molecules depends largely upon their sizes and electric charges, which therefore influence the position at which precipitation occurs. Moreover, the precipitation is confined to the zone in which antigen and antibody are present in the relative proportions required for the formation of visible flocculation Thus in a complex system containing several antigens and their corresponding antibodies, multiple precipitation zones distinctly separated from each other may occur. It is this separation of the reaction zones of different antigenic components of a virus which gives the diffusion techniques their particular value, although the interpretation of multiple zoning is often a matter of great difficulty. The methods are specially suited for the detection of minor antigenic components of a virus and of minor cross relationships between different viruses and virus strains.

With three standard monkey antisera and a number of paired sera from human cases of poliomyelitis there was a general rough correlation, and the paired sera (acute and convalescent) showed the same trends of rise of titre by both neutralisation and precipitation tests. No evidence of the existence of group antigens shared by viruses of different types was obtained.

LE BOUVIER, SCHWERDT AND SCHAFFER (1957) attempted to define the significance of the major and minor precipitation bands by testing different fractions obtained from purified virus concentrates. The major band was produced by a fraction containing nearly all the infective virus particles and with an RNA content of 25 to 30 per cent. A different band, probably corresponding with the minor band given by many crude concentrates, was produced by a fraction which had very little RNA and contained mostly non-infective particles, less uniform and flatter than virus particles.

(2) Microscopic Method

A microscopic gel diffusion technique, originally evolved by GRASSET, PONGRATZ AND BRECHBUHLER (1956) for the study of venoms and other antigens has now been shown by them to be applicable to the investigation of poliomyelitis virus flocculation (GRASSET, BONIFAS AND PONGRATZ, 1958). Microscopic slides are coated with a thin layer of agar gel in which 2mm. diameter holes, 4mm. apart, are punched which hold 4cmm. volumes of the reagents. The slides are incubated within sealed glass chambers in an atmosphere saturated with water vapour. Precipitation lines appear in 24 hours or less, indeed further incubation is stated, without further comment, to result in their regression. The most probable explanation of such regression is diffusion of the precipitate, after its formation in the zone of optimal proportions of antigen and antibody. The flocculation lines are best observed either by dark ground microscopy or by naked eye after fixation and staining of the preparations. The antigens used were essentially the same as those employed by SMITH ET AL. in the micro-tube method, being fifty-fold concentrations of fluids from virus-infected cell cultures. They were found to remain fully active after two months' storage at 4°C. Instability of the antigens, in the sense of spontaneous aggregation of particles, which has so far been a major difficulty with the micro-tube method, is not mentioned but in any case would be unlikely to preclude their use in gel diffusion flocculation.

In spite of these limiting factors, recent work has indicated the great potentialities of plate gel diffusion precipitation as a research tool in virology. BODON (1955) showed that it could be used to identify the classical O, A and C types of foot and mouth disease virus. His work was confirmed by BROWN AND CRICK (1957) who extended the investigation to include several of the newer foot and mouth disease virus types and also the Indiana and New Jersey types of vesicular stomatitis virus. Unlike BODON however, they found that any virus type usually gives two distinct precipitation zones with homotypic serum, the exact significance of which remains undetermined. They also reported the occasional occurrence of slight reactions with heterotypic sera. This raises the interesting question as to whether these viruses contain a common group antigen. In their paper, BROWN and CRICK mention a personal communication to the effect that MOOSBRUGGER, SPUHLER and MEYER have used the technique for the titration of antibody in the sera of cattle convalescent from infection with foot and mouth disease.

The recent studies of LE BOUVIER (1957) and of LE BOUVIER, SCHWERDT AND SCHAFFER (1957), with poliomyelitis viruses have elicited several further points of great interest. There seems to be no doubt that the elementary bodies of these very small viruses can diffuse *in toto* to produce a major precipitation band with the antibodies of a homotypic serum. Minor bands may also be produced by virus components with different diffusion rates but their occurrence and relative positions depend upon both the particular virus concentrates and the particular antisera used, doubtless because of the variability in the concentrations of the reactants which these materials contain. Thus pairs of bands were usually obtained with hyperimmune monkey sera but clearly defined minor bands were not seen with human sera. This may reflect either a fundamental difference between the immunological reactions of different animal species, or, more probably, merely a difference between the immunogenic stimuli to which the monkeys and human beings had been subjected. In any case the anomaly clearly indicates a line along which further exploration would be profitable. LE BOUVIER also showed that the method can be used for precipitin assay because there is a linear relationship between log serum concentration and distance of the major precipitation band from the serum reservoir, provided that virus dose is kept constant. The practical importance of this depends to some extent on the correlation between precipitation and virus neutralisation titres of sera.

altogether no matter how long the mixtures are incubated. BOYD (1941) described two basic patterns of isochronic flocculation, an R pattern, common with rabbit antisera, in which inhibition occurs only in the zone of antigen excess and an H pattern, given by horse and some rabbit antisera, in which it occurs in the zones of both antigen and antibody excess.

As was only to be expected, similar inhibition zones have been found to occur in virus flocculation reactions. This introduces a considerable risk that absence of any reaction in diagnostic tests may be falsely interpreted. From the studies of BELYAVIN it appears that

Table I. Influenza Flocculation: chessboard titration

Time of incubation at 37°C	Initial dilns. of PR 8 virus antigen.	Initial dilution of rabbit antiserum.*							Controls	
		1/5	1/10	1/20	1/40	1/80	1/160	1/320	NRS* 1/20	Saline
½ hr	1/1	T−	T−	S+	?tr	—	—	—	—	—
	1/2	S+	S+	S	tr+	?tr	—	—	—	—
	1/4	S−	S−	S−	tr+	tr	—	—	—	—
	1/8	tr	tr	tr	tr	?tr	—	—	—	—
	1/16	?tr	—	—	—	—	—	—	—	—
	1/32	—	—	—	—	—	—	—	—	—
	1/64	—	—	—	—	—	—	—	—	—
1½ hrs	1/1	T	T	T	S−	—	—	—	—	—
	1/2	T	T	T−	T−	tr	—	—	—	—
	1/4	S+	S+	S+	S	S−	—	—	—	—
	1/8	S−	S−	S−	S−	tr+	tr	—	—	—
	1/16	tr+	tr+	tr+	tr+	tr	tr	—	—	—
	1/32	?tr	?tr	—	—	—	—	—	—	—
	1/64	—	—	—	—	—	—	—	—	—
5 hrs.	1/1	T	T	T	S	—	—	—	—	—
	1/2	T	T	T	T	tr+	—	—	—	—
	1/4	T	T	T	T	T−	?tr	—	—	—
	1/8	T−	T−	T−	T−	T−	S−	—	—	—
	1/16	S+	S+	S+	S+	S	S−	tr	—	—
	1/32	tr+	tr+	tr+	tr+	tr+	tr+	tr	—	—
	1/64	tr	tr	tr	tr	?tr	?tr	—	—	—

* Heterologous antibodies and inhibitors removed by absorption and heat inactivation.
NRS = Normal rabbit serum.

The results reported by GRASSET AND HIS CO-WORKERS are of a preliminary nature and it is difficult to assess the potentialities of their technique at the present juncture. They report the occurrence of double-precipitation lines with monospecific monkey antisera against *virus types 1 and 2, but only a single line with type 3 reagents. Both* acute and convalescent sera of paralytic cases of poliomyelitis gave strong reactions. Reactions were also obtained with pooled gamma globulins against all three virus types, and against type 1 virus with seven out of twelve pre-vaccination and ten out of twelve post-vaccination sera, from human beings immunised with Salk vaccine. Sera of animals immunised with Salk vacine gave precipitation lines against the three monotypic antigens.

Such results if confirmed would indicate that this technique has a degree of sensitivity considerably greater than that of any other method evolved as yet for virological flocculation work. This might actually constitute a disadvantage for rapid diagnostic application, for without the introduction of quantitative antibody assay the significance of reactions obtained with human sera would be difficult to determine. It is also suggested that the results indicate the existence of group precipitating antibodies quite distinct from type specific neutralising antibodies, but in view of the complexity of poliomyelitis flocculation reactions revealed by plate diffusion, much further evidence is needed to substantiate this view.

The many advantages of the method, including its speed, economy in the use of reagents and, not least, the fixation of reactions in permanent form for subsequent reference and comparisons, invite its *further exploration with other viruses. One would expect that in* some cases it could be readily adapted to serve as a useful diagnostic tool.

V. Flocculation Inhibition Zones

In precipitation reactions with pure soluble antigens and strictly monospecific sera, speed of flocculation depends not only upon the concentration of the reagents but also upon their relative proportions. This results in patterns of isochronic flocculation, demonstrable by chess board titrations in which sufficiently extensive dilution ranges of both antigen and antibody are included. Gross excess of either reagent over the proportions which yield most rapid flocculation, the so called optimal proportions, may indeed prevent particulation

tion zones occur in regions of both excess antigen and excess antibody. A typical chess board titration done by the micro tube method is illustrated in table II. This shows an isochronic flocculation pattern of the BOYD H type. In line titrations with constant antigen dose an excess antibody inhibition zone, if it exists, is immediately apparent, whereas there is nothing to show whether the serum dilution end point is a true antibody end point or is due to inhibition from antigen excess. This may be one reason why poliomyelitis flocculation requires the use of highly concentrated virus antigens, for with unconcentrated virus suspensions the excess antibody inhibition zone may extend over a very extensive range of dilutions of the antiserum. It is certainly not the only reason, however, why virus concentration is necessary. Probably of more importance is the fact that with such small viruses, unconcentrated culture fluids do not contain sufficient bulk of antigen for the antigen-antibody lattices which form to become visible. The need for concentrated antigens in the gel diffusion techniques supports this view*.

The suggestion that the different patterns of isochronic flocculation, obtained with the influenza and poliomyelitis viruses respectively, may represent a fundamental difference between the flocculation behaviour of different viruses must be regarded with caution Unfortunately these are the only viruses for which we posses relevant information and the poliomyelitis virus concentrates employed were not comparable, in respect of purity, with the influenza virus suspensions of BELYAVIN for reasons stated above The effects of impurities in flocculation systems have not been systematically investigated, except in the case of the flocculation inhibitors discussed below, but there is no doubt that they may modify the flocculation pattern produced by pure reagents. Mention has already been made of the importance which early workers attached to the extraction of ether soluble material from vaccine lymph and of the failure of high titre lung suspensions from mice infected with influenza virus to give specific flocculation. In recent experiments with poliomyelitis virus antigens their further purification has resulted in some instances in abolition of the excess antibody inhibition zone without any apparent reduction of antigenic potency (SMITH, SHEFFIELD and CHURCHER,

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* Since this paper was written the possibility of obtaining flocculation with high titre unconcentrated poliomyelitis fluids has been demonstrated independently in two different laboratories (Unpublished observations)

with influenza viruses and rabbit antisera inhibition occurs only in the region of antigen excess conforming to BOYD'S R pattern of isochronic flocculation. In an extensive series of full chessboard titrations of different virus strains and rabbit antisera, BELYAVIN has never encountered inhibition from excess antibody, that reported in his first paper being now known to be due to the presence of inhibitory substances (personal communication). This is exemplified by the experiment shown in table I. Flocculation with serum dilutions higher than 1 in 40 was completely prevented in the mixtures which contained the undiluted antigen, but there was no sign of inhibition from excess antibody even in mixtures containing minimal amounts of antigen. Obviously the use of this virus suspension, in a constant antigen dose titration, would fail to detect the presence of antibodies in a relatively weak serum with an antibody content one-eightieth or less of that of the particular serum used in this experiment. But total flocculation might be obtained with such weak antisera provided that the dose of antigen used was small enough.

Poliomyelitis antigens and antisera of rabbits immunised by the same technique as was used by BELYAVIN give flocculation behaviour of different character (SMITH ET AL, unpublished). Here inhibi-

Table II. Chess board titration of poliomyelitis virus type 2, and rabbit immune serum by the micro tube method

Antigen diln	Initial dilution of antiserum								Antigen + Saline controls
	1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1280	
1/1	−	−	±	+	++	+	−	−	−
1/2	−	−	−	±	+	+	±	−	−
1/4	−	−	−	−	+	+	+	−	−
1/8	−	−	−	−	−	+	±	±	−
1/16	−	−	−	−	−	−	±	±	−
1/32	−	−	−	−	−	−	−	±	−

Table III Differentiation between Haemagglutinin and Flocculation Inhibition of Influenza Virus

Inhibitors			
Treatment	Haemagglutination		Flocculation
	α	β	
56°C	+	—	—
RDE	—	+	+
Trypsin	—	?—	—
Sod Citrate	+	+	—

+ indicates retention of inhibitory activity
— indicates loss of inhibitory activity
RDE — Receptor Destroying Enzyme

show that each of these can be selectively removed by appropriate treatments. As BELYAVIN points out, this indicates that flocculation inhibitor probably combines with receptor sites on the virus surface which are distinct from the haemagglutinin receptors and offers a new line of approach to the analysis of virus surface structure.

The exact nature of the flocculation inhibitors is not yet known They function, as do the haemagglutinin inhibitors, essentially as nonspecific "blocking" antibodies which combine with antigen receptors without visible effect, possibly because they are univalent. The behaviour of the flocculation inhibitors, however, cannot be attributed to univalency Some influenza virus strains can yield either "stable" or "unstable" antigen suspensions, the latter being directly flocculated by normal sera unless the serum inhibitors are removed by heat inactivation or other treatment. Against virus suspensions in this "unstable" state, therefore, the flocculation inhibitors function as non-specific flocculating antibodies.

Similar substances for other viruses have not yet been reported, though it will be surprising if none such exist Their importance as regards diagnostic applications of flocculation reactions is obvious, fortunately their removal from sera is not likely to present any insuperable difficulty.

unpublished observations). This suggests that the relatively crude poliomyelitis antigens may contain inhibitors similar to the serum flocculation inhibitors discussed below, but it is difficult to explain the phenomenon on this basis. Unless the serum used is itself inhibitory the presence of inhibitor in the antigen preparation should not cause inhibition only in mixtures with the largest serum doses, but should either prevent flocculation through the whole serum dilution range or merely enhance the antigen excess inhibition zone. So far no definite evidence of the participation of serum inhibitors in poliomyelitis flocculation has been obtained.

Until the phenomena of inhibition zoning have been more fully investigated with several different viruses little reliance can be placed on results obtained by line titrations in which a constant dose of one reagent is used with serial dilutions of the other reagent. For the investigation of any new system extensive chess board titrations are essential and, even for rough screening tests, experience with poliomyelitis reagents has shown that varying doses of both antigen and antiserum may be required for the detection of antibodies in either a strong or a weak immune serum.

VI. Flocculation Inhibitors

Virus haemagglutination inhibitors occur in various biological materials, especially in animal sera. Those affecting influenza virus haemagglutination have been very extensively investigated and in some cases have been isolated in pure form and chemically characterised. They are non-specific in the immunological sense and their removal is essential for the full analysis of the specific antigen antibody reactions. Recognition of the existence of similar flocculation inhibitors comes from BELYAVIN's studies of influenza virus flocculation (BELYAVIN, 1955, 1956, 1957). Like the haemagglutination inhibitors they are widely distributed in animal sera and different species, and different individuals of the same species show wide variations of serum inhibitor content.

Particular interest attaches to the fact that some at least of the inhibitors of flocculation are clearly distinguishable from those which inhibit haemagglutination. Two types of the latter have been described, namely α and β inhibitors, and both may occur together with flocculation inhibitor in a single serum. The data presented in table III

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VII. Conclusion

Virus antibody flocculation reactions have not yet been sufficiently explored to permit more than a very tentative opinion of their relative importance in virology. The reasons for past neglect of this serological approach are no longer operative for many viruses can now be grown in cell culture with relatively simple nutrient media and technological resources for virus concentration are now widely available. The little work so far done gives clear indication that the extension of flocculation studies to other animal viruses cannot fail to yield much information about their antigenic constitution and structure. Applications of the reactions in routine diagnostic work are likely to remain largely empirical for some considerable time but may, none the less, introduce the simplicity, economy and precision which have hitherto been lacking in the field of diagnostic virology.

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